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I, JONNE YABSLEY, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PR 1279 for a patent by MONASH UNIVERSITY, NATIONAL UNIVERSITY OF SINGAPORE and HADASIT MEDICAL RESEARCH SERVICES & DEVELOPMENT CO LTD filed on 06 November 2000.

WITNESS my hand this
Fifteenth day of June 2001

J R Yabsley

JONNE YABSLEY
TEAM LEADER EXAMINATION
SUPPORT AND SALES

AUSTRALIA
Patents Act 1990

PROVISIONAL SPECIFICATION

Invention Title: EMBRYONIC STEM CELLS AND NEURAL PROGENITOR CELLS DERIVED THEREFROM (2)

Applicant: MONASH UNIVERSITY, NATIONAL UNIVERSITY OF SINGAPORE AND HADASIT MEDICAL RESEARCH SERVICES & DEVELOPMENT CO. LTD.

The invention is described in the following statement:

EMBRYONIC STEM CELLS AND NEURAL PROGENITOR CELLS DERIVED THEREFROM (2)

The present invention relates to undifferentiated human embryonic stem cells, methods of cultivation and propagation and production of differentiated cells. In particular it relates to the production of human ES capable of yielding somatic differentiated cells *in vitro*, as well as committed progenitor cells such as neural progenitor cells capable of giving rise to mature somatic cells including neural cells and/or glial cells and uses thereof.

INTRODUCTION

The production of human embryonic stem cells which can be either maintained in an undifferentiated state or directed to undergo differentiation into extraembryonic or somatic lineages *in vitro* allows for the study of the cellular and molecular biology of early human development, functional genomics, generation of differentiated cells from the stem cells for use in transplantation or drug screening and drug discovery *in vitro*.

In general, stem cells are undifferentiated cells which can give rise to a succession of mature functional cells. For example, a haematopoietic stem cell may give rise to any of the different types of terminally differentiated blood cells. Embryonic stem (ES) cells are derived from the embryo and are pluripotent, thus possessing the capability of developing into any organ, cell type or tissue type or, at least potentially, into a complete embryo.

The development of mouse ES cells in 1981 (Evans and Kaufman, 1981; Martin, 1981) provided the paradigm, and, much of the technology, for the

development of human ES cells. Development of ES cells evolved out of work on mouse teratocarcinomas, (tumours arising in the gonads of a few inbred strains), which consist of a remarkable array of somatic tissues juxtaposed together in a disorganised fashion. Classical work on teratocarcinomas established their origins from germ cells in mice, and provided the concept of a stem cell (the embryonal carcinoma or EC cell) which could give rise to the multiple types of tissue found in the tumours (Kleinsmith and Pierce, 1964; review, Stevens, 1983). The field of teratocarcinoma research (review, Martin, 1980) expanded considerably in the 70's, as the remarkable developmental capacity of the EC stem cell became apparent following the generation of chimaeric mice by blastocyst injection of EC cells, and investigators began to realise the potential value of cultured cell lines from the tumours as models for mammalian development. EC cells however had limitations. They often contained chromosomal abnormalities, and their ability to differentiate into multiple tissue types was often limited.

Since teratocarcinomas could also be induced by grafting blastocysts to ectopic sites, it was reasoned that it might be possible to derive pluripotential cell lines directly from blastocysts rather than from tumours, as performed in 1981 by Gail Martin and Martin Evans independently. The result was a stable diploid cell line which could generate every tissue of the adult body, including germ cells. Teratocarcinomas also develop spontaneously from primordial germ cells in some mouse strains, or following transplantation of primordial germ cells to ectopic sites, and in 1992 Brigid Hogan and her colleagues reported the direct derivation of EG cells from mouse primordial germ cells (Matsui et al., 1992). These EG cells have a developmental capacity very similar to ES cells.

Testicular teratocarcinomas occur spontaneously in humans, and pluripotential cell lines were also developed from these (review, Andrews,

1988). Two groups reported the derivation of cloned cell lines from human teratocarcinoma which could differentiate in vitro into neurons and other cell types (Andrews et al., 1984, Thompson et al., 1984). Subsequently, cell lines were developed which could differentiate into tissues representative of all three embryonic germ layers (Pera et al., 1989). As analysis of the properties of human EC cells proceeded, it became clear that they were always aneuploid, usually (though not always) quite limited in their capacity for spontaneous differentiation into somatic tissue, and different in phenotype from mouse ES or EC cells.

The properties of the pluripotent cell lines developed by Pera et al. (1989) are as follows:

- Express SSEA-3,SSEA-4, TRA 1-60, GCTM-2, alkaline phosphatase, Oct-4
- Grow as flat colonies with distinct cell borders
- Differentiate into derivatives of all three embryonic germ layers
- Feeder cell dependent (feeder cell effect on growth not reconstituted by conditioned medium from feeder cells or by feeder cell extracellular matrix)
- Highly sensitive to dissociation to single cells, poor cloning efficiency even on a feeder cell layer
- Do not respond to Leukemia Inhibitory Factor

These studies of human EC cells essentially defined the phenotype of primate pluripotential stem cells.

Derivation of primate ES cells from the rhesus monkey blastocyst and later from that of the marmoset (Thomson et al., 1995, 1996)has been described. These primate cell lines were diploid, but otherwise they closely resembled their nearest counterpart, the human EC cell. The implication of the monkey work and the work on human EC cells was that

a pluripotent stem cell, which would be rather different in phenotype from a mouse ES cell, could likely be derived from a human blastocyst.

Bongso and coworkers (1994) reported the short term culture and maintenance of cells from human embryos fertilised *in vitro*. The cells isolated by Bongso and coworkers had the morphology expected of pluripotent stem cells, but these early studies did not employ feeder cell support, and it was impossible to achieve long term maintenance of the cultures.

James Thomson and coworkers (1998) derived ES cells from surplus blastocysts donated by couples undergoing treatment for infertility. The methodology used was not very different from that used 17 years earlier to derive mouse ES stem cells. The trophectoderm, thought to be inhibitory to ES cell establishment, was removed by immunosurgery, the inner cell mass was plated on to a mouse embryonic fibroblast feeder cell layer, and following a brief period of attachment and expansion, the resulting outgrowth was disaggregated and replated onto another feeder cell layer. There were no significant departures from mouse ES protocols in the media or other aspects of the culture system and a relatively high success rate was achieved. The phenotype of the cells was similar to that outlined above in the human EC studies of Pera et al.

In the studies of Thomson et al. on monkey and human ES cells, there was no evidence that the cells showed the capacity for somatic differentiation *in vitro*. Evidence for *in vitro* differentiation was limited to expression of markers characteristic of trophoblast and endoderm formation (production of human chorionic gonadotrophin and alphafoetoprotein). It is not possible to state whether the cells found producing alphafetoprotein represent extraembryonic (yolk sac) endoderm or definitive (embryonic) endoderm though the former is far more likely.

Thus an essential feature for any human ES cell line to be of practical use, namely the production of differentiated somatic cells *in vitro* as seen in previous studies of human EC cells, was not demonstrated in the monkey or human ES cell studies.

Much attention recently has been devoted to the potential applications of stem cells in biology and medicine, the properties of pluripotentiality and immortality are unique to ES cells and enable investigators to approach many issues in human biology and medicine for the first time. ES cells potentially can address the shortage of donor tissue for use in transplantation procedures, particularly where no alternative culture system can support growth of the required committed stem cell. However, it must be noted that almost all of the wide ranging potential applications of ES cell technology in human medicine-basic embryological research, functional genomics, growth factor and drug discovery, toxicology, and cell transplantation are based on the assumption that it will be possible to grow ES cells on a large scale, to introduce genetic modifications into them, and to direct their differentiation. Present systems fall short of these goals, but there are indications of progress to come. The identification of novel factors driving pluripotential stem cell growth or stem cell selection protocols to eliminate the inhibitory influence of differentiated cells, both offer a way forward for expansion and cloning of human ES cells.

The mammalian nervous system is a derivative of the ectodermal germ layer of the postimplantation embryo. During the process of axis formation, it is thought that inductive signals elaborated by several regions of the embryo (the anterior visceral endoderm and the early gastrula organiser) induce the pluripotent cells of the epiblast to assume an anterior neural fate (Beddington and Robertson, 1999). The molecular identity of the factors elaborated by these tissues which direct neurogenesis is unknown, but there is strong evidence from lower

vertebrates that antagonists of the Wnt and BMP families of signalling molecules may be involved.

Embryonic stem cells are pluripotent cells which are thought to correspond to the epiblast of the periimplantation embryo. Mouse ES cells are able to give rise to neural tissue *in vitro* either spontaneously or during embryoid body formation. The neural tissue often forms in these circumstances in amongst a mixture of a range of cell types. Alteration of the conditions of culture, or subsequent selection of neural cells from this mixture, has been used to produce relatively pure populations of neuronal cells from differentiating cultures of ES cells (eg Li et al., 1998). These neuronal cells have been used in experimental models to correct various deficits in animal model systems (review, Svendsen and Smith, 1999). The same has not yet been achieved with human ES cell derived neurons, though neuronal cells have been derived from human embryonal carcinoma cells which were induced to differentiate using retinoic acid. These EC cells were subsequently shown to correct deficits in experimental models of CNS disease.

A suitable source of human ES derived neurons would be desirable since their availability would provide real advantages for basic and applied studies of CNS development and disease. Controlled differentiation of human ES cells into the neural lineage will allow experimental dissection of the events during early development of the nervous system, and the identification of new genes and polypeptide factors which may have a therapeutic potential such as induction of regenerative processes. Additional pharmaceutical applications may include the creation of new assays for toxicology and drug discovery, such as high-throughput screens for neuroprotective compounds. Generation of neural progenitors from ES cells *in vitro* may serve as an unlimited source of cells for tissue

reconstruction and for the delivery and expression of genes in the nervous system.

It is an object of the invention to overcome or at least alleviate some of the problems of the prior art.

SUMMARY OF THE INVENTION

In one aspect of the present invention, there is provided a purified preparation of undifferentiated human embryonic stem cells capable of proliferation *in vitro* and differentiation to neural progenitor cells, neuron cells and/or glial cells.

Preferably the undifferentiated ES cells have the potential to differentiate into neural progenitor cells, neuron cells and/or glial cells when subjected to differentiating conditions.

More preferably, the undifferentiated ES cells are capable of maintaining an undifferentiated state when cultured on a fibroblast feeder layer.

In another aspect of the present invention there is provided an undifferentiated human embryonic stem cell wherein the cell is immunoreactive with markers for human pluripotent stem cells including SSEA-4, GCTM-2 antigen, TRA 1-60 and wherein said cell may differentiate under differentiating conditions to neural cells. Preferably, the cells express the transcription factor Oct-4 as demonstrated by RT-PCR. More preferably, the cells maintain a diploid karyotype during prolonged cultivation *in vitro*.

In another aspect there is provided an undifferentiated cell line capable of differentiation into neural progenitor cells, neuronal and glial cells and preferably produced by a method of the present invention.

In another aspect there is provided a differentiated committed progenitor cell line that may be cultivated for prolonged periods and give rise to large quantities of progenitor cells.

In another aspect there is provided a differentiated committed progenitor cell line capable of differentiation into mature neurons and/or glial cells.

In another aspect, there is provided a neural progenitor cell, neuron cell and/or a glial cell differentiated *in vitro* from an undifferentiated embryonic stem cell. There is also provided a committed neural progenitor cell capable of giving rise to mature neuron cells.

Preferably, the undifferentiated cell line is preserved by preservation methods such as cryopreservation. Preferably the method of cryopreservation is a method highly efficient for use with embryos such as vitrification. Most preferably, the method includes the Open Pulled Straw (OPS) vitrification method.

In another aspect, there is provided a neural progenitor cell capable of differentiating into glial cells, including astrocytes and oligodendrocytes.

In another aspect, there is provided a neural progenitor cell capable of transdifferentiation into other cell lineages, to generate stem cells and differentiated cells of non-neuronal phenotype, such as hematopoietic stem cells.

In a further aspect of the present invention, there is provided a method of preparing undifferentiated human embryonic stem cells for differentiation into neural progenitor cells, said method including:

obtaining an *in vitro* fertilised human embryo and growing the embryo to a blastocyst stage of development;

removing inner cells mass (ICM) cells from the embryo;

culturing ICM cells under conditions which do not induce extraembryonic differentiation and cell death and promote proliferation of undifferentiated cells; and

recovering stem cells.

In a further preferred embodiment of the present invention there is provided a method of preparing undifferentiated human embryonic stem cells for differentiation into neural progenitor cells, said method including:

obtaining an *in vitro* fertilised human embryo;

removing inner cell mass (ICM) cells from the embryo;

culturing ICM cells on a fibroblast feeder layer to obtain proliferation of embryonic stem cells; and

recovering stem cells from the feeder layer.

In a further embodiment of the invention, the method further includes:

replacing the stem cells from the fibroblast feeder layer onto another fibroblast feeder layer; and

culturing the stem cells for a period sufficient to obtain proliferation of morphologically undifferentiated stem cells.

In another aspect of the invention the method further includes propagating the undifferentiated stem cells.

In another aspect of the invention there is provided a method of inducing somatic differentiation of stem cells *in vitro* into progenitor cells said method comprising :

obtaining undifferentiated stem cells; and

providing a differentiating signal under conditions which are non-permissive for stem cell renewal, do not kill cells and reduces unidirectional differentiation toward extraembryonic lineages.

In a preferred embodiment of the present invention, there is provided a method of inducing somatic differentiation of stem cells *in vitro* into progenitor cells, said method comprising:

obtaining undifferentiated stem cells; and

culturing said cells for a prolonged period and at high density on a fibroblast feeder cell layer to induce differentiation.

In another preferred embodiment of the present invention, there is provided a method of inducing somatic differentiation of stem cells *in vitro* into progenitor cells, said method comprising:

obtaining undifferentiated stem cells; and

transferring said cells into serum free media to induce differentiation.

In an additional aspect of the invention method may be used for directing stem cells to differentiate toward a somatic lineage. Furthermore, the method allows the establishment of a pure preparation of progenitor cells from a desired lineage and facilitate the establishment of a pure somatic progenitor cell line.

This invention provides a method that generates an *in vitro* model of controlled differentiation of ES cells towards the neural lineage. The model, and the cells that are generated along the pathway of neural

differentiation may be used for the study of the cellular and molecular biology of human neural development, for the discovery of genes, growth factors, and differentiation factors that play a role in neural differentiation and regeneration, for drug discovery and for the development of screening assays for teratogenic, toxic and neuroprotective effects.

In a further aspect of the invention there is provided a neural progenitor cell, a neuronal cell and a glial cell that may be used for cell therapy and gene therapy.

FIGURES

Figure 1 shows phase contrast micrographs of ES cells and their differentiated progeny. A, inner cell mass three days after plating. B, colony of ES cells. C, higher magnification of an area of an ES cell colony. D, an area of an ES cell colony undergoing spontaneous differentiation during routine passage. E, a colony four days after plating in the absence of a feeder cell layer but in the presence of 2000 units/ml human LIF undergoing differentiation in its periphery,. F, neuronal cells in a high density culture. Scale bars: A and C, 25 microns; B and E, 100 microns; D and F, 50 microns.

Figure 2 shows marker expression in ES cells and their differentiated somatic progeny. A, ES cell colony showing histochemical staining for alkaline phosphatase. B. ES cell colony stained with antibody MC-813-70 recognising the SSEA-4 epitope. C, ES cell colony stained with antibody TRA1-60. D, ES cell colony stained with antibody GCTM-2. E, high density culture, cell body and processes of a cell stained with anti-neurofilament 68kDa protein. F, high density culture, cluster of cells and network of processes emanating from them stained with antibody against neural cell adhesion molecule. G, high density culture, cells showing

cytoplasmic filaments stained with antibody to muscle actin. H, high density culture, cell showing cytoplasmic filaments stained with antibody to desmin. Scale bars: A, 100 microns; B-D, and F, 200 microns; E, G and H, 50 microns.

Figure 3 shows RT-PCR analysis of gene expression in ES cells and their differentiated derivatives. All panels show 1.5% agarose gels stained with ethidium bromide. A, expression of Oct-4 and b-actin in ES stem cells and high density cultures. Lane 1, 100 bpDNA ladder. Lane 2, stem cell culture, b-actin. Lane 3, stem cell culture, Oct-4. Lane 4, stem cell culture, PCR for Oct-4 carried out with omission of reverse transcriptase. Lane 5, high density culture, b-actin. Lane 6, high density culture, Oct-4. Lane 7, high density culture, PCR for Oct-4 carried out with omission of reverse transcriptase. b-actin band is 200 bp and Oct-4 band is 320 bp. B, expression of nestin and Pax-6 in neural progenitor cells. Left lane, 100bp DNA ladder; lane 1, b-actin in HX 142 neuroblastoma cell line (positive control for nestin PCR); lane 2, b-actin in neural progenitor cells; lane 3, nestin in HX 142 neuroblastoma cell line; lane 4, nestin in neural progenitor cells; lane 5, nestin PCR on same sample as lane 4 without addition of reverse transcriptase; lane 6, Pax-6; lane 7, Pax-6 PCR on same sample as line 6 without addition of reverse transcriptase. Nestin band is 208 bp, Pax-6 is 274 bp. C, expression of glutamic acid decarboxylase in cultures of neurons. Left lane, 100bp DNA ladder; lane 1, b-actin; lane 2, b-actin PCR on same sample as lane 1 without addition of reverse transcriptase; lane 3, glutamic acid decarboxylase; lane 4 glutamic acid decarboxylase on same sample as lane 3 without addition of reverse transcriptase. Glutamic acid decarboxylase band is 284 bp. D, expression of GABA Aa2 receptor. Left lane, 100bp DNA ladder; lane 1, b-actin; lane 2, GABA Aa2 receptor; lane 3, PCR without addition of reverse transcriptase. GABA Aa2 receptor subunit band is 471 bp.

Figure 4 shows histology of differentiated elements found in teratomas formed in the testis of SCID mice following inoculation of HES-1 or HES-2 colonies. A, cartilage and squamous epithelium, HES-2. B, neural rosettes, HES-2. C, ganglion, gland and striated muscle, HES-1. D, bone and cartilage, HES-1. E, glandular epithelium, HES-1. F, ciliated columnar epithelium, HES-1. Scale bars: A-E, 100 microns; F, 50 microns.

Figure 5 shows phase contrast microscopy and immunochemical analysis of marker expression in neural progenitor cells isolated from differentiating ES cultures. A, phase contrast image of a sphere formed in serum-free medium. B-D, indirect immunofluorescence staining of spheres, 4 hours after plating on adhesive substrate, for N-CAM, nestin, and vimentin respectively. In C and D, cells at the base of the sphere were placed in plane of focus to illustrate filamentous staining; confocal examination revealed that cells throughout the sphere were decorated by both antibodies. Scale bar is 100 microns in all panels.

Figure 6 shows phase contrast appearance and marker expression in cultures of neurons derived from progenitor cells shown in Figure 5. A, phase contrast micrograph of differentiated cells emanating from a sphere plated onto adhesive surface. B-H, indirect immunofluorescence microscopy of differentiated cells decorated with antibodies against 200 kDa neruofilament protein (B), 160 kDa neurofilament protein (C), MAP2a+b (D), glutamate (E), synaptophysin (F), glutamic acid decarboxylase (G) and (-tubulin (H). Scale bars: A,;B, 100 microns; C, 200mircons; D, 20 microns; E and F, 10 microns; G, 20 microns; H, 25 microns.

Figure 7 shows neural precursors proliferating as a monolayer on a plastic tissue culture dish in the presence of EGF and bFGF. These monolayer

cultures of proliferating cells were obtained after prolonged cultivation (2-3 weeks) of the spheres in the presence of growth factors without sub-culturing.

Figure 8 shows phase contrast appearance of a cultured consisting entirely of differentiated neuronal cells.

Figure 9 shows phase contrast appearance of a sphere that is formed 72 hours after the transfer of a clump of undifferentiated ES cells into serum free medium (Scale bar 100 microns).

Figure 10 shows linear correlation between the volume of spheres and the number of progenitor cells within a sphere. Spheres at passage level 11-13 of various diameters were dissaggregated into single cell suspension and the number of cells per sphere was counted.

Figure 11 shows indirect immunofluorescence staining of a sphere , 4 hours after plating on adhesive substrate, for N-CAM. The sphere was generated by direct transfer of undifferentiated ES cells into serum free medium and propagation of the resulting spheres for 5 passages. (Scale bar 100 microns).

Figure 12 shows indirect immunofluorescence membraneous staining for N-CAM of single cells at the periphery of a sphere 4 hours after plating on adhesive substrate. The sphere was generated by direct transfer of undifferentiated ES cells into serum free medium and propagation of the resulting spheres for 5 passages. (Scale bar 25 microns).

Figure 13 shows indirect immunofluorescence staining of a spheres 4 hours after plating on adhesive substrate for the intermediate filament nestin. Cells at the base of the sphere were placed in plane of focus to

illustrate filamentous staining. The sphere was generated by direct transfer of undifferentiated ES cells into serum free medium and propagation of resulting spheres for 5 passages. (Scale bar 25 microns).

Figure 14 shows indirect immunofluorescence microscopy of a differentiated cell decorated with antibodies against the oligodendrocyte progenitor marker O4. (Scale bar 12.5 microns).

Figure 15 shows indirect immunofluorescence staining of a sphere 4 hours after plating on adhesive substrate for the intermediate filament vimentin. Cells at the base of the sphere were placed in plane of focus to illustrate filamentous staining. The sphere was generated by direct transfer of undifferentiated ES cells into serum free medium and propagation of resulting spheres for 7 passages. (Scale bar 25 microns).

DESCRIPTION OF THE INVENTION

In one aspect of the present invention there is provided a purified preparation of human undifferentiated embryonic stem cells capable of proliferation *in vitro* and differentiation to neural progenitor cells, neuron cells and/or glial cells.

Proliferation *in vitro* may include cultivation of the cells for prolonged periods. The cells are substantially maintained in an undifferentiated state. Preferably the cells are maintained under conditions which do not induce cell death or extraembryonic differentiation.

Preferably, they are capable of maintaining an undifferentiated state when cultured on a fibroblast feeder layer preferably under non-differentiating

conditions. Preferably the fibroblast feeder layer does not induce extraembryonic differentiation.

More preferably the cells have the potential to differentiate *in vitro* when subjected to differentiating conditions. Most preferably the cells have the capacity to differentiate *in vitro* into a wide array of somatic lineages.

The promotion of stem cells capable of being maintained in an undifferentiated state *in vitro* on one hand, and which are capable of differentiation *in vitro* into extraembryonic and somatic lineages on the other hand, allows for the study of the cellular and molecular biology of early human development, functional genomics, generation of differentiated cells from the stem cells for use in transplantation or drug screening and drug discovery *in vitro*.

Once the cells are maintained in the undifferentiated state, they may be differentiated to mature functional cells. The embryonic stem cells are derived from the embryo and are pluripotent and have the capability of developing into any organ or tissue type. Preferably the tissue type is selected from the group including blood cells, neural cells or muscle cells. Most preferably they are neural cells.

In another aspect of the present invention there is provided an undifferentiated human embryonic stem cell wherein the cell is immunoreactive with markers for human pluripotent stem cells including SSEA-4, GCTM-2 antigen, TRA 1-60 and wherein said cell can differentiate, under differentiating conditions to neural cells. Preferably, the cells express specific transcription factors such as Oct-4 as demonstrated by RT-PCR, or methods of analysis of differential gene expression, microarray analysis or related techniques. More preferably the cells maintain a diploid karyotype during prolonged cultivation *in vitro*.

Preferably, the stem cell will constitute a purified preparation of an undifferentiated stem cell line. More preferably, the stem cell line is a permanent cell line, distinguished by the characteristics identified above. They preferably have normal karyotype along with the characteristics identified above. This combination of defining properties will identify the cell lines of the invention regardless of the method used for their isolation.

Methods of identifying these characteristics may be by any method known to the skilled addressee. Methods such as (but not limited to) indirect immunoflourescence or immunocytochemical staining may be carried out on colonies of ES cells which are fixed by conventional fixation protocols then stained using antibodies against stem cell specific antibodies and visualised using secondary antibodies conjugated to fluorescent dyes or enzymes which can produce insoluble colored products. Alternatively, RNA may be isolated from the stem cells and RT-PCR or Northern blot analysis carried out to determine expression of stem cell specific genes such as Oct-4.

In a preferred embodiment the undifferentiated cells form tumours when injected in the testis of immunodeprived SCID mice. These tumours include differentiated cells representative of all three germ layers. The germ layers are preferably endoderm, mesoderm and ectoderm. Preferably, once the tumours are established, they may be disassociated and specific differentiated cell types may be identified or selected by any methods available to the skilled addressee. For instance, lineage specific markers may be used through the use of fluorescent activated cell sorting (FACS) or other sorting method or by direct micro dissection of tissues of interest. These differentiated cells may be used in any manner. They may be cultivated *in vitro* to produce large numbers of differentiated cells which could be used for transplantation or for use in drug screening for example.

In another aspect there is provided a differentiated committed progenitor cell line capable of differentiation and propagation into mature neurons and/or glial cells. The undifferentiated cells may differentiate *in vitro* to form neural progenitor cells, neuron cells and/or glial cells.

In another aspect, there is provided a neural progenitor cell differentiated *in vitro* from an undifferentiated embryonic stem cell. There is also provided a committed neural progenitor cell capable of giving rise to mature neuron cells.

In another aspect, there is provided a neural progenitor cell capable of differentiating into glial cells, including astrocytes and oligodendrocytes. The glial cells include microglial cells and radial glial cells.

In another aspect, there is provided a neural progenitor cell capable of transdifferentiation into other cell lineages, to generate stem cells and differentiated cells of non-neuronal phenotype, such as hematopoietic stem cells.

These cells may be obtained by somatic differentiation of human ES cells, identified by neural markers. These cells may be isolated in pure form from differentiating ES cells, *in vitro*, and propagated *in vitro*. They may be induced to under go differentiation to mature neurons and/or glial cells.

The cells may undergo differentiation *in vitro* to yield neural progenitor cells or neuron cells as well as extraembryonic cells, such differentiation being characterised by novel gene expression characteristic of specific lineages as demonstrated by immunocytochemical or RNA analysis. Characterisation may be obtained by using expression of genes characteristic of pluripotent cells or particular lineages. Preferably,

differential expression of Oct-4 may be used to identify stem cells from differentiated cells. Otherwise, the presence or absence of expression of other genes characteristic of pluripotent stem cells or other lineages may include Genesis, GDF-3 or Cripto. Analysis of these gene expressions may create a gene expression profile to define the molecular phenotype of an ES cell, a committed progenitor cell, or a mature differentiated cell of any type. Such analysis of specific gene expression in defined populations of cells from ES cultures is called cytomics. Methods of analysis of gene expression profiles include RT-PCR, methods of differential gene expression, microarray analysis or related techniques.

Differentiating cultures of the stem cells secrete human chorionic gonadotrophin (hCG) and α-fetoprotein (AFP) into culture medium, as determined by enzyme-linked immunosorbent assay carried out on culture supernatants. Hence this may also serve as a means of identifying the differentiated cells.

The differentiated cells forming neural progenitor cells, neuron cells and/or glial cells may also be characterised by expressed markers characteristic of differentiating cells. The *in vitro* differentiated cell culture may be identified by detecting molecules such as markers of primitive neuroectoderm, neural cell adhesion molecule, neuro-filament proteins, monoclonal antibodies such as MAP2a, b, glutamate, synaptophysin, glutamic acid decarboxylase, β-tubulin, β-tubulin III, glial fibrillary acidic protein (GFAP), gal C and O4.

In a further aspect of the invention, there is provided a method of preparing undifferentiated human embryonic stem cells for differentiation into neural progenitor cells, said method including:

obtaining an *in vitro* fertilised human embryo and growing the embryo to a blastocyst stage of development;

removing inner cells mass (ICM) cells from the embryo;

culturing ICM cells under conditions which do not induce extraembryonic differentiation and cell death, and promote proliferation of undifferentiated stem cells; and

recovering stem cells.

The stem cells will be undifferentiated cells and can be induced to differentiate when a differentiating signal is applied.

In a preferred embodiment of the present invention there is provided a method of preparing undifferentiated human embryonic stem cells for differentiation into neural progenitor cells, said method including:

obtaining an *in vitro* fertilised human embryo;

removing inner cells mass (ICM) cells from the embryo;

culturing ICM cells on a fibroblast feeder layer to obtain proliferation of embryonic stem cells; and

recovering stem cells from the feeder layer.

Embryonic stem cells (ES) are derived from the embryo. These cells are undifferentiated and have the capability of differentiation to a variety of cell types. The "embryo" is defined as any stage after fertilization up to 8 weeks post conception. It develops from repeated division of cells and includes the stages of a blastocyst stage which comprises an outer trophectoderm and an inner cell mass (ICM).

The embryo required in the present method may be an *in vitro* fertilised embryo or it may be an embryo derived by transfer of a somatic cell or cell nucleus into an enucleated oocyte of human or non human origin which is then activated and allowed to develop to the blastocyst stage.

The embryo may be fertilised by any *in vitro* methods available. For instance, the embryo may be fertilised by using conventional insemination, or intracytoplasmic sperm injection.

An embryo that is recovered from cryopreservation is also suitable. An embryo that has been cryopreserved at any stage of development is suitable. Preferably embryos that were cryopreserved at the zygote or cleavage stage are used. Any method of cryopreservation of embryos may be used. It is preferred that a method producing high quality (good morphological grade) embryos is employed.

It is preferred that any embryo culture method is employed but it is most preferred that a method producing high quality (good morphological grade) blastocysts is employed. The high quality of the embryo can be assessed by morphological criteria. Most preferably the inner cell mass is well developed. These criteria can be assessed by the skilled addressee.

Following insemination, embryos may be cultured to the blastocyst stage. Embryo quality at this stage may be assessed to determine suitable embryos for deriving ICM cells. The embryos may be cultured in any medium that maintains their survival and enhances blastocyst development.

Preferably, the embryos are cultured in droplets under pre-equilibrated sterile mineral oil in IVF-50 or Scandinavian 1 (S1) or G1.2 medium (Scandinavian IVF). Preferably the incubation is for two days. If IVF-50 or S1 is used, on the third day, an appropriate medium such as a mixture of 1:1 of IVF-50 and Scandinavian-2 medium (Scandinavian IVF) may be used. From at least the fourth day, a suitable medium such as G2.2 or Scandinavian-2 (S2) medium may be used solely to grow the embryos to

blastocyst stage (blastocysts). Preferably, only G2.2 medium is used from the fourth day onwards.

In a preferred embodiment, the blastocyst is subjected to enzymatic digestion to remove the zona pellucida or a portion thereof. Preferably the blastocyst is subjected to the digestion at an expanded blastocyst stage which may be approximately on day 6. Generally this is at approximately six days after insemination.

Any protein enzyme may be used to digest the zona pellucida or portion thereof from the blastocyst. Examples include pronase, acid Tyrodes solution, and mechanical methods such as laser dissection.

Preferably, Pronase is used. The pronase may be dissolved in PBS and G2 or S2 medium. Preferably the PBS and Scandinavian-2 medium is diluted 1:1. For digestion of zone pellucida from the blastocyst, approximately 10 units/ml of Pronase may be used for a period sufficient to remove the zona pellucida. Preferably approximately 1 to 2 mins, more preferably 1-1.5 mins is used.

The embryo (expanded blastocyst) may be washed in G2.2 or S2 medium, and further incubated to dissolve the zona pellucida. Preferably, further digestion steps may be used to completely dissolve the zona. More preferably the embryos are further incubated in pronase solution for 15 seconds. Removal of the zona pellucida thereby exposes the trophectoderm.

In a preferred embodiment of the invention the method further includes the following steps to obtain the inner cell mass cell, said steps including:

treating the embryo to dislodge the trophectoderm of the embryo or a portion thereof;

washing the embryo with a G2.2 or S2 medium to dislodge the trophectoderm or a portion thereof; and

obtaining inner cell mass cells of the embryo.

Having had removed the zona pellucida, the ICM and trophectoderm become accessible. Preferably the trophectoderm is separated from the ICM. Any method may be employed to separate the trophectoderm from the ICM. Preferably the embryo (or blastocyst devoid of zona pellucida) is subjected to immuno-surgery. Preferably it is treated with an antibody or antiserum reactive with epitopes on the surface of the trophectoderm. More preferably, the treatment of the embryo, (preferably an embryo at the blastocyst stage devoid of zona pellucida) is combined with treatment with complement. The antibody and/or antiserum and complement treatment may be used separately or together. Preferred combinations of antibody and/or antiserum and complement include anti-placental alkaline phosphatase antibody and Baby Rabbit complement (Serotec) or anti-human serum antibody (Sigma) combined with Guinea Pig complement (Gibco).

Preferably the antibodies and complement are diluted in G2.2 or S2 medium. The antibodies and complement, excluding anti-placental alkaline phosphate (anti-AP) are diluted 1:5 whereas anti-AP antibody is diluted 1:20 with S-2 medium.

Preferably the embryo or blastocyst (preferably having the zona pellucida removed) is subjected to the antibody before it is subjected to the complement. Preferably, the embryo or blastocyst is cultured in the antibody for a period of approximately 30 mins.

Following the antibody exposure, it is preferred that the embryo is washed. Preferably it is washed in G2.2 or S2 medium. The embryo or blastocyst

preferably is then subjected to complement, preferably for a period of approximately 30 mins.

G2.2 or S2 (Scandinavian-2) medium is preferably used to wash the embryo or blastocyst to dislodge the trophectoderm or a portion thereof. Dislodgment may be by mechanical means. Preferably the dislodgment is by pipetting the blastocyst through a small bore pipette.

The ICM cells may then be exposed and ready for removal and culturing. Culturing of the ICM cells may be conducted on a fibroblast feeder layer. In the absence of a fibroblast feeder layer, the cells will differentiate. Leukaemia inhibitory factor (LIF) has been shown to replace the feeder layer in some cases and maintain the cells in an undifferentiated state. However, this seems to only work for mouse cells. For human cells, high concentrations of LIF were unable to maintain the cells in an undifferentiated state in the absence of a fibroblast feeder layer.

The conditions which do not induce extraembryonic differentiation and cell death may include cultivating the embryonic stem cells on a fibroblast feeder layer which does not induce extraembryonic differentiation and cell death.

Mouse or human fibroblasts are preferably used. They may be used separately or in combination. Human fibroblasts provide support for stem cells, but they create a non-even and sometimes non-stable feeder layer. However, they may combine effectively with mouse fibroblasts to obtain an optimal stem cell growth and inhibition of differentiation.

The cell density of the fibroblast layer affects its stability and performance. A density of approximately 25,000 human and 70,000 mouse cells per cm2 is most preferred. Mouse fibroblasts alone are used at 75,000-

100,000/cm2. The feeder layers are preferably established 6-48 hours prior to addition of ES cells.

Preferably the mouse or human fibroblast cells are low passage number cells. The quality of the fibroblast cells affects their ability to support the stem cells. Embryonic fibroblasts are preferred. For mouse cells, they may be obtained from 135 day old foetuses. Human fibroblasts may be derived from embryonic or foetal tissue from termination of pregnancy and may be cultivated using standard protocols of cell culture.

The guidelines for handling the mouse embryonic fibroblasts may include minimising the use of trypsin digestion and avoidance of overcrowding in the culture. Embryonic fibroblasts that are not handled accordingly will fail to support the growth of undifferentiated ES cells. Each batch of newly derived mouse embryonic fibroblasts is tested to confirm its suitability for support and maintenance of stem cells.

Fresh primary embryonic fibroblasts are preferred in supporting stem cell renewal as compared to frozen-thawed fibroblasts. Nevertheless, some batches will retain their supportive potential after repeated freezing and thawing. Therefore each fresh batch that has proved efficient in supporting ES cells renewal is retested after freezing and thawing. Batches that retain their potential after freezing and thawing are most preferably used. Batches are tested to determine suitability for the support of stem cell renewal, the induction of somatic differentiation or the induction of extraembryonic differentiation.

Fresh primary embryonic fibroblasts are preferred in supporting stem cell renewal and/or induction of somatic differentiation as compared to frozen-thawed fibroblasts. Nevertheless, some batches will retain their supportive potential after repeated freezing and thawing. Therefore each fresh batch

that has proved efficient in supporting ES cells renewal and/or induction of somatic differentiation is retested after freezing and thawing. Batches that retain their potential after freezing and thawing are most preferably used.

Some mouse strains yield embryonic fibroblasts which are more suitable for stem cell maintenance and induction of somatic differentiation than those of other strains. For example, fibroblasts derived from inbred 129/Sv or CBA mice or mice from a cross of 129/Sv with C57/Bl6 strains have proven highly suitable for stem cell maintenance.

Isolated ICM masses may be plated and grown in culture conditions suitable for human stem cells.

It is preferred that the feeder cells are treated to arrest their growth. Several methods are available. It is preferred that they are irradiated or are treated with chemicals such as mitomycin C which arrests their growth. Most preferably, the fibroblast feeder cells are treated with mitomycin C (Sigma).

The fibroblast feeder layer maybe generally plated on a gelatin treated dish. Preferably, the tissue culture dish is treated with 0.1% gelatin.

The fibroblast feeder layer may also contain modified fibroblasts. For instance, fibroblasts expressing recombinant membrane bound factors essential for stem cell renewal may be used. Such factors may include for example human multipotent stem cell factor.

Inner cell mass cells may be cultured on the fibroblast feeder layer and maintained in an ES medium. A suitable medium is DMEM (GIBCO, without sodium pyruvate, with glucose 4500mg/L) supplemented with 20% FBS (Hyclone, Utah), (betamercaptoethanol - 0.1mM (GIBCO), non

essential amino acids - NEAA 1% (GIBCO), glutamine 2mM. (GIBCO), and penicillin 50µ/ml, streptomycin 50µg/ml (GIBCO). In the early stages of ES cell cultivation, the medium maybe supplemented with human recombinant leukemia inhibitory factor hLIF preferably at 2000µ/ml. However, LIF generally is not necessary. Any medium may be used that can support the ES cells.

The ES medium may be further supplemented with soluble growth factors which promote stem cell growth or survival or inhibit stem cell differentiation. Examples of such factors include human multipotent stem cell factor, or embryonic stem cell renewal factor.

The isolated ICM may be cultured for at least six days. At this stage, a colony of cells develops. This colony is comprised principally of undifferentiated stem cells. They may exist on top of differentiated cells. Isolation of the undifferentiated cells may be achieved by chemical or mechanical means or both. Preferably mechanical isolation and removal by a micropipette is used. Mechanical isolation may be combined with a chemical or enzymatic treatment to aid with dissociation of the cells, such as Ca^{2+}/Mg^{2+} free PBS medium or dispase.

In a further preferred embodiment of the invention, the method further includes:

replating the stem cells from the fibroblast feeder layer onto another fibroblast feeder layer; and

culturing the stem cells for a period sufficient to obtain proliferation of morphologically undifferentiated stem cells.

A further replating of the undifferentiated stem cells is performed. The isolated clumps of cells from the first fibroblast feeder layer may be

replated on fresh human/mouse fibroblast feeder layer in the same medium as described above.

Preferably, the cells are cultured for a period of 7-14 days. After this period, colonies of undifferentiated stem cells may be observed. The stem cells may be morphologically identified preferably by the high nuclear/cytoplasmic ratios, prominent nucleoli and compact colony formation. The cell borders are often distinct and the colonies are often flatter than mouse ES cells. The colonies resemble those formed by pluripotent human embryonal carcinoma cell lines such as GCT 27 X-1.

In another embodiment of the invention, the method further includes propagating the undifferentiated stem cells. The methods of propagation may initially involve removing clumps of undifferentiated stem cells from colonies of cells. The dispersion is preferably by chemical or mechanical means or both. More preferably, the cells are washed in a Ca^{2+}/Mg^{2+} free PBS or they are mechanically severed from the colonies or a combination of these methods or any known methods available to the skilled adressee. In these methods, cells may be propagated as clumps of about 100 cells about every 7 days.

In the first method, Ca^{2+}/Mg^{2+} free PBS medium may be used to reduce cell-cell attachments. Following about 15-20 minutes, cells gradually start to dissociate from the monolayer and from each other and desired size clumps can be isolated. When cell dissociation is partial, mechanical dissociation using the sharp edge of the pipette may assist with cutting and the isolation of the clumps.

An alternative chemical method may include the use of an enzyme. The enzyme may be used alone or in combination with a mechanical method. Preferably, the enzyme is dispase.

An alternative approach includes the combined use of mechanical cutting of the colonies followed by isolation of the subcolonies by dispase. Cutting of the colonies may be performed in PBS containing Ca^{2+} and Mg^{2+}. The sharp edge of a micropipette may be used to cut the colonies to clumps of about 100 cells. The pipette may be used to scrape and remove areas of the colonies. The PBS is preferably changed to regular equilibrated human stem cell medium containing dispase (Gibco) 10 mg/ml and incubated for approximately 5minutes at 37°C in a humidified atmosphere containing 5% CO_2. As soon as the clumps detached they may be picked up by a wide bore micro-pipette, washed in PBS containing Ca^{2+} and Mg^{2+} and transferred to a fresh fibroblast feeder layer.

The fibroblast feeder layer may be as described above.

Undifferentiated embryonic stem cells have a characteristic morphology as described above. Other means of identifying the stem cells may be by cell markers or by measuring expression of genes characteristic of pluripotent cells.

Examples of genes characteristic of pluripotent cells or particular lineages may include (but are not limited to) Oct-4 and Pax-6, nestin and vimentin as markers of stem cells and neuronal precursors respectively. Other genes characteristic of stem cells may include Genesis, GDF-3 and Cripto. Such gene expression profiles may be attained by any method including RT-PCR, methods of differential gene expression, microarray analysis or related techniques.

Preferably the stem cells may be identified by being immunoreactive with markers for human pluripotent stem cells including SSEA-4, GCTM-2

antigen, TRA 1-60. Preferably the cells express the transcription factor Oct-4. The cells also maintain a diploid karyotype.

Preferably the neural progenitor cells are identified by expressed markers of primitive neuroectoderm such as polysialyated N-CAM intermediate filament proteins such as nestin and vimentin and the transcription factor Pax-6, Neurons may be identified by structural markers such as β-tubulin, β-tubulin III, the 68kDa and the 200kDa neurofilament proteins. Mature neurons may also be identified by the 160 kDa neurofilament proteins, Map-2a, b and synaptophysin, glutamate, GABA biosynthesis and receptor subunits characteristic of GABA minergic neurons (GABA A(2). Glial progenitors may be identified by the marker O4.

The stem cells may be further modified at any stage of isolation. They may be genetically modified through introduction of vectors expressing a selectable marker under the control of a stem cell specific promoter such as Oct-4. Some differentiated progeny of embryonic stem cells may produce products which are inhibitory to stem cell renewal or survival. Therefore selection against such differentiated cells, facilitated by the introduction of a construct such as that described above, may promote stem cell growth and prevent differentiation.

The stem cells may be genetically modified at any stage with markers so that the markers are carried through to any stage of cultivation. The markers may be used to purify the differentiated or undifferentiated stem cell population at any stage of cultivation.

Genetic construct may be inserted to undifferentiated or differentiated cells at any stage of cultivation. The genetically modified cells may be used after transplantation to carry and express genes in target organs in the course of gene therapy.

Progress of the stem cells and their maintenance in a differentiated or undifferentiated stage may be monitored in a quantitative fashion by the measurement of stem cell specific secreted products into the culture medium or in fixed preparations of the cells using ELISA or related techniques. Such stem cell specific products might include the soluble form of the CD30 antigen or the GCTM-2 antigen or they may be monitored as described above using cell markers or gene expression.

In another aspect of the invention there is provided a method of inducing somatic differentiation of stem cells *in vitro* into progenitor cells said method comprising:

obtaining undifferentiated stem cells; and

providing a differentiating signal under conditions which are non-permissive for stem cell renewal, do not kill cells and/or reduces unidirectional differentiation toward extraembryonic lineages.

The undifferentiated cell lines of the present invention may be cultured indefinitely until a differentiating signal is given.

In the presence of a differentiation signal, undifferentiated ES cells in the right conditions will differentiate into derivatives of the embryonic germ layers (endoderm, mesoderm and ectoderm) such as neuron tissue, and/or extraembryonic tissues. This differentiation process can be controlled.

This method is useful for directing stem cells to differentiate toward a somatic lineage. Furthermore, the method allows the establishment of a pure preparation of progenitor cells from a desired lineage and the elimination of unwanted differentiated cells from other lineages. The method facilitates the establishment of a pure somatic progenitor cell line.

The method may be used to derive a pure preparation of a variety of somatic progenitors such as but not limited to mesodermal progenitors (such as hemangioblast or hematopoietic stem cells) and neural progenitors. Preferably the method is used to derive neural progenitors.

Conditions for obtaining differentiated cultures of somatic cells from embryonic stem cells are those which are non-permissive for stem cell renewal, but do not kill stem cells or drive them to differentiate exclusively into extraembryonic lineages. A gradual withdrawal from optimal conditions for stem cell growth favours somatic differentiation. The stem cells are initially in an undifferentiated state and can be induced to differentiate.

In a preferred embodiment of the present invention, there is provided a method of inducing somatic differentiation of stem cells *in vitro* into progenitor cells, said method comprising:

obtaining undifferentiated stem cells; and

culturing said cells for prolonged periods and at high density on a fibroblast feeder cell layer to induce differentiation.

In another preferred embodiment of the present invention, there is provided a method of inducing somatic differentiation of stem cells *in vitro* into progenitor cells, said method comprising:

obtaining undifferentiated stem cells; and

transferring said cells into serum free media to induce differentiation.

The stem cells may be undifferentiated stem cells and derived from any source or process which provides viable undifferentiated stem cells. The

methods described above for retrieving stem cells from embryos is most preferred.

In these preferred aspects, the conditions of culturing the cells at high density on a fibroblast feeder cell layer or transferring to a serum free medium are intended to be non-permissive for stem cell renewal or cause uni-directional differentiation toward extraembryonic lineages.

Generally the presence of a fibroblast feeder layer will maintain these cells in an undifferentiated state. This has been found to be the case with the cultivation of mouse and human ES cells. However, without being restricted by theory, it has now become evident that the type and handling of the fibroblast feeder layer is important for maintaining the cells in an undifferentiated state or inducing differentiation of the stem cells. Suitable fibroblast feeder layers are discussed above.

Somatic differentiation *in vitro* of the ES cell lines is a function of the period of cultivation following subculture, the density of the culture, and the fibroblast feeder cell layer. It has been found that somatic differentiation is morphologically apparent and demonstrable by immunochemistry approximately 14 days following routine subcultivation as described above in areas of the colony which are remote from direct contact with the feeder cell layer (in contrast to areas adjacent to the feeder cell layer where rapid stem cell growth is occurring such as the periphery of a colony at earlier time points after subcultivation), or in cultures which have reached confluence. Depending upon the method of preparation and handling of the mouse embryo fibroblasts, the mouse strain from which the fibroblasts are derived, and the quality of a particular batch, stem cell renewal, extraembryonic differentiation or somatic differentiation may be favoured.

Once a suitable fibroblast cell line is selected, it may be used as a differentiation inducing fibroblast feeder layer to induce the undifferentiated stem cells to differentiate into a somatic lineage or multiple somatic lineages. These may be identified using markers or gene expression as described above. Preferably the fibroblast feeder layer does not induce extraembryonic differentiation and cell death.

The modulation of stem cell growth by appropriate use of fibroblast feeder layer and manipulation of the culture conditions thus provides an example whereby somatic differentiation may be induced *in vitro* concomitant with the limitation of stem cell renewal without the induction of widespread cell death or extraembryonic differentiation.

Other manipulations of the culture conditions such as culturing in various compositions of serum free medium may be used to arrest stem cell renewal without causing stem cell death or unidirectional extraembryonic differentiation, thereby favouring differentiation of somatic cells.

Differentiation may also be induced by cultivating to a high density in monolayer or on semi-permeable membranes so as to create structures mimicing the postimplantation phase of human development, or any modification of this approach. Cultivation in the presence of cell types representative of those known to modulate growth and differentiation in the vertebrate embryo (eg. endoderm cells or cells derived from normal embyronic or neoplastic tissue) or in adult tissues (eg. bone marrow stromal preparation) may also induce differentiation, modulate differentiation or induce maturation of cells within specific cell lineage so as to favour the establishment of particular cell lineages.

Chemical differentiation may also be used to induce differentiation. Propagation in the presence of soluble or membrane bound factors known

to modulate differentiation of vertebrate embryonic cells, such as bone morphogenetic protein-2 or antagonists of such factors, may be used.

Applicants have found that Oct-4 is expressed in stem cells and down-regulated during differentiation and this strongly indicates that stem cell selection using drug resistance genes driven by the Oct-4 promoter will be a useful avenue for manipulating human ES cells. Directed differentiation using growth factors, or the complementary strategy of lineage selection coupled with growth factor enhancement could enable the selection of populations of pure committed progenitor cells from spontaneously differentiating cells generated as described here.

Genetic modification of the stem cells or further modification of those genetically modified stem cells described above may be employed to control the induction of differentiation. Genetic modification of the stem cells so as to introduce a construct containing a selectable marker under the control of a promoter expressed only in specific cell lineages, followed by treatment of the cells as described above and the subsequent selection for cells in which that promoter is active may be used.

Once the cells have been induced to differentiate, the various cell types, identified by means described above, may be separated and selectively cultivated. Preferably neural progenitor cells are selected. These progenitors are capable of differentiating into neuron cells and/or glial cells. More preferably, they will differentiate into neuron cells and/or glial cells in the absence of other differentiated cells in the extra embryonic lineage

Selective cultivation means isolation of specific lineages of progenitors or mature differentiated cells from mixed populations preferably appearing under conditions unfavourable for stem cell growth and subsequent

propagation of these specific lineages. Selective cultivation may be used to isolate populations of mature cells or populations of lineage specific committed progenitor cells. Isolation may be achieved by various techniques in cell biology including the following alone or in combination: microdissection; immunological selection by labelling with antibodies against epitopes expressed by specific lineages of differentiated cells followed by direct isolation under flourescence microscopy, panning, immunomagnetic selection, or selection by flow cytometry; selective conditions favouring the growth or adhesion of specific cell lineages such as exposure to particular growth or extracellular matrix factors or selective cell-cell adhesion; separation on the basis of biophysical properties of the cells such as density; disaggregation of mixed populations of cells followed by isolation and cultivation of small clumps of cells or single cells in separate culture vessels and selection on the basis of morphology, secretion of marker proteins, antigen expression, growth properties, or gene expression; lineage selection using lineage specific promoter constructs driving selectable markers or other reporters.

The derivation of neural progenitors from ES cells, and even further more, the establishment of a pure neural progenitor cell line is described below as proof of the above principles. The following description is illustrative of neural progenitor cells as somatic cells differentiated from stem cells and should not be taken as a restriction on the generality of the invention. It should be noted that the method may be used to derive a pure preparation of a variety of somatic progenitors such as but not limited to mesodermal progenitors such as hemangioblast or hematopoietic stem cells or neural progenitors.

The establishment of neural progenitor cells from embryonic stem cells and more preferably a pure preparation of neural progenitor cells and

even more preferably a neural progenitor cell line may be achieved byany one or combination of the following approaches.

In one preferred approach, somatic differentiation of ES cells is induced by prolonged culture of ES cells to high density on an appropriate fibroblast feeder layer that prevents unidirectional differentiation towards extraembryonic lineage and promotes somatic differentiation. Once the cells have been induced to differentiate toward somatic lineages, areas which are destined to give rise to clusters of mainly neural progenitor cells may be mechanically separated based on characteristic morphological features as described above, and replated in serum-free medium, whereupon they form spherical structures.

Any serum free medium may be used. Preferably NS-A (Euroclone) or DMEM/F12 (Gibco) is used. More preferably NS-A or DMEM/F12 supplemented with N2 or B27 (Gibco) is used. Most preferably DMEM/F12 supplemented with B27 is used.

In the presence of an appropriate supplement of growth factors such as but not limited to, EGFand basic FGF to the serum free medium, the neural progenitors may be cultivated and expanded to establish a cell line. The growth factors inhibit further differentiation of the progenitor cells and promote their proliferation.

The culture in the serum free medium is selective and therefore limits prolonged proliferation of other types of differentiated cells such as the progeny of the extraembryonic lineage that may coexist in the culture. Therefore the cultivation in these selective conditions may be used to establish a purified cell line of neural progenitors.

The progenitors may be cultivated as spheres or as a monolayer. Subculturing may be conducted mechanically. Scraping is preferred to propagate monolayer cultures. However, any mechanical method such as tituration or cutting may be used to subculture the spheres. Most preferably the spheres are sliced into smaller clumps. The progenitors may be expanded to produce a large number of cells.

In another preferred approach, the method involves the transfer of undifferentiated stem cells into culture conditions that on one hand direct differentiation toward a desired somatic lineage, which is the neural lineage in this case, while on the other hand are selective and therefore limit both the differentiation toward unwanted lineages (such as extraembryonic lineages) as well as the survival of differentiated cells from these lineages. Such culture conditions include the transfer into serum free media (as described above). The serum free media promotes differentiation towards the neuroectodermal lineage (and possibly other non-neural lineages such as mesoderm). The serum free media may limit the growth and survival of unwanted cells such as those from the extraembryonic lineage.

In a further preferred embodiment of the invention, the method allows the establishment of a pure progenitor cell line from the desired lineage.

Growth factors may be added to the medium and may promote the proliferation and the cultivation of the desired somatic progenitors such as neural progenitors. The selective culture conditions further eliminate during cultivation, unwanted differentiated cells from other lineages such as extraembryonic lineages. The method may be used to derive a pure preparation and/or a pure cell line of a variety of somatic progenitors including, but not limited to, neural progenitor and mesodermal progenitors such as hemangioblast or hematopoietic stem cells.

Preferably, in the derivation of a purified cell line of neural progenitors, clumps of undifferentiated stem cells may be transferred into plastic tissue culture dishes containing serum free medium. The serum free medium induces the differentiation of the ES cells initialy towards ectoderm and then towards the neuroectodermal lineage.

Any serum free medium may be used. Preferably NS-A medium (Euroclone) or DMEM/F12 is used. More preferably the serum free medium is supplemented with N2 or B27 (Gibco). The clusters of undifferentiated stem cells turn into round spheres within approximately 24 hours after transfer (Figure 9).

The serum free medium may be further supplemented with basic FGF and EGF to promote proliferation of neural progenitors in an undifferentiated state. The progenitors may be cultivated under these conditions for prolonged periods. The selective conditions that are induced by the serum free medium result in a gradual purification and elimination of other differentiated cell types during cultivation.

The progenitors may be cultivated as spheres or as a monolayer. Subculturing may be conducted mechanically. Scraping is preferred to propagate monolayer cultures. However, any mechanical method known to the skilled addressee such as tituration or cutting may be used to subculture the spheres. Most preferably the spheres are sliced into smaller clumps. The progenitors may be expanded to produce a large number of cells .

The progenitors that are generated directly from undifferentiated stem cells have similar properties to the neural progenitors that are generated from differentiating stem cells colonies. They express the same markers of

primitive neuroectoderm, such as NCAM, the intermediate filament protein nestin, Vimentin and the transcription factor Pax-6.They generate differentiated neural cells with similar morphology and marker expression after plating on appropriate substrate and withdrawal of growth factors.

In another aspect of the invention, there is provided a method of inducing somatic cells from embryonic stem cell derived somatic progenitors, said method comprising:

obtaining a source of embryonic stem cell derived somatic progenitor cells grown in the presence of a serum free media and growth factors;

culturing the progenitor cells on an adhesive substrate; and

withdrawing growth factors from the media to allow for induction of the somatic cells.

The source of embryonic stem cell derived progenitor cells may be from any source. However, they are preferably established by the methods described above.

The somatic cells may preferably be neurons or oligodendrocyte progenitor cells. Preferably, the somatic progenitors are neural progenitors.

Any adhesive substrate may be used. More preferably, poly lysine and laminin or poly lysine and fibronectin are used.

For inducing neurons, it is preferred to use poly lysine and laminin.

Upon plating of neural progenitors on an appropriate substrate such as poly lysine and laminin, and withdrawal of growth factors from the serum free medium, differentiated cells grow out of the spheres as a monolayer

and acquire morphology of mature neurons and expression of markers such as the 160 kd neurofilament protein , Map-2AB, synaptophysin, Glutamate, GABA biosynthesis and receptor subunits characteristic of GABA minergic neurons (GABA A(2) which are characteristic of mature neurons.

In a preferred embodiment, the method for inducing neurons further includes culturing the somatic progenitor cells, preferably undifferentiated neural progenitor cells, or differentiating neuronal progenitors in the presence of retinoic acid.

Retinoic acid has been found to further induce differentiation toward mature neurons.

For inducing oligodendocyte progenitors, it is preferred to use poly lysine and fibronectin.

The establishment of oligodendrocyte cells indicates the potential of the neural precursors to differentiate towards the glial lineage.

In a preferred embodiment, the method for inducing oligodendocyte progenitor cells further includes culturing the somatic progenitor cells, preferably undifferentiated neural progenitor cells, in the presence of PDGF and basic FGF.

In yet another preferred embodiment, the method for inducing oligodendocyte progenitor cells further includes culturing the somatic progenitor cells, preferably undifferentiated neural progenitor cells, in the presence of T3. Preferably the cells are then grown in the absence of growth factor.

These culture conditions direct the neural progenitors to turn into glial progenitors. This is followed by plating the progenitors on poly lysine and fibronectin and further culture in the presence of the growth factors and T3 followed by culture in the presence of T3 without growth factor supplementation. Without being limited by theory, it is postulated that the growth factors such as bFGF and PDGF facilitate proliferation and spreading of the glial promoters while fibronectin and T3 induce the differentiation toward and along the oligodendrocyte lineage.

Oligodendrocyte may be also induced by plating the neural progenitors on poly lysine and fibronectin and further culture in the presence of bFGF and T3 followed by culture in the presence of T3 and PDGF.

The progenitor cells that are derived by the method that is described above may be used to generate differentiated cells from other lineages. The spheres of progenitors may include in addition to neural progenitors more primitive cells such as primitive ectodermal cells. By manipulation of the culture conditions these primitive cells may generate all somatic cell types.

The present invention provides a method that generates an *in vitro* model of controlled differentiation of ES cells towards the neural lineage. The model, and the cells that are generated along the pathway of neural differentiation may be used for the study of the cellular and molecular biology of human neural development, for the discovery of genes, growth factors, and differentiation factors that play a role in neural differentiation and regeneration. The model, and the cells that are generated along the pathway of neural differentiation may be used for drug discovery and for the development of screening assays for teratogenic, toxic and neuroprotective effects.

In a further aspect of the invention, there is provided a method of producing large quantities of differentiated and undifferentiated cells.

In a further aspect of the invention there is provided a neural progenitor cell, a neuronal cell and a glial cell that may be used for cell therapy and gene therapy.

Genetically manipulated neural progenitor cells or neuronal cell or glial cells may be used after transplantation as a vector to carry and express desired genes at target organs.

In another aspect of the present invention, there is provided a committed progenitor cell line. The progenitor cell line may be expanded to produce large quantities of progenitor cells, neural progenitor cells, neuronal cells, mature neuronal cells and glial cells.

In another aspect of the invention, there are provided committed neural progenitor cells capable of self renewal or differentiation into one or limited number of somatic cell lineages, as well as mature differentiated cell produced by the methods of the present invention.

Expansion of the committed progenitor cells may be useful when the number of progenitors that may be derived from ES cells is limited. In such a case, expansion of the progenitors may be useful for various applications such as the production of sufficient cells for transplantation therapy, for the production of sufficient RNA for gene discovery studies etc. For example, by using the techniques described above, expansion of progenitor cells from ten spheres for ten passages may generate $50x10^6$ cells that would be sufficient for any application.

These observations on cells of the neural lineage establish the principle that by using the techniques described, committed progenitor cells may be isolated, from embryonic stem cell cultures propagated, expanded, purified and further induced to produce fully differentiated cells.

In a further aspect of the invention, there is provided a method of producing large quantities of differentiated and undifferentiated cells.

In another aspect there is provided a differentiated committed progenitor cell line that may be cultivated for prolonged periods and give rise to large quantities of progenitor cells and fully differentiated cells.

The neural progenitor cells or other committed progenitor cells derived by the method described above may be used to generate differentiated cells from other lineages by transdifferentiation.

In another aspect there is provided a differentiated committed progenitor cell line capable of differentiation into mature neurons and/or glial cells. Preferably the progenitor cell is a neural progenitor cell.

In another aspect there is provided an undifferentiated cell line capable of differentiation into neural progenitor cells produced by the method of the present invention.

Specific cell lines HES-1 and HES-2 were isolated by the procedures described above and have the properties described above.

In another aspect of the invention there is provided a cell composition including a human differentiated or undifferentiated cell capable of differentiation into neural progenitor cells preferably produced by the method of the present invention, and a carrier.

The carrier may be any physiologically acceptable carrier that maintains the cells. It may be PBS or ES medium.

The differentiated or undifferentiated cells may be preserved or maintained by any methods suitable for storage of biological material. Vitrification of the biological material is the preferred method over the traditional slow-rate freezing methods.

Effective preservation of ES cells is highly important as it allows for continued storage of the cells for multiple future usage. Although traditional slow freezing methods, commonly utilised for the cryo-preservation of cell lines, may be used to cryo- preserve undifferentiated or differentiated cells, the efficiency of recovery of viable human undifferentiated ES cells with such methods is extremely low. ES cell lines differ from other cell lines since the pluripotent cells are derived from the blastocyst and retain their embryonic properties in culture. Therefore, cryo-preservation using a method which is efficient for embryos is most appropriate. Any method which is efficient for cryo-preservation of embryos may be used. Preferably, vitrification method is used. More preferably the Open Pulled Straw (OPS) vitrification method previously described by Vajta, G. et al (1998) Molecular Reproduction and Development, 51, 53-58, is used for cryopreserving the undifferentiated cells. More preferably, the method described by Vajta, G. et al (1998) Cryo-Letters, 19, 389 - 392 is employed. Generally, this method has only been used for cryopreserving embryos.

The differentiated or undifferentiated cells may be used as a source for isolation or identification of novel gene products including but not limited to growth factors, differentiation factors or factors controlling tissue regeneration, or they may be used for the generation of antibodies against

novel epitopes. The cell lines may also be used for the development of means to diagnose, prevent or treat congenital diseases.

Much attention recently has been devoted to the potential applications of stem cells in biology and medicine. The properties of pluripotentiality and immortality are unique to ES cells and enable investigators to approach many issues in human biology and medicine for the first time. ES cells potentially can address the shortage of donor tissue for use in transplantation procedures, particularly where no alternative culture system can support growth of the required committed stem cell. ES cells have many other far reaching applications in human medicine, in areas such as embryological research, functional genomics, identification of novel growth factors, and drug discovery, and toxicology.

While the potential applications of neural stem cells derived from adult or embryonic CNS are considerable, there may be real advantages to neural progenitor cells derived from ES cell cultures.

ES cell lines derived from a patients own tissue via somatic cell nuclear transfer would produce neuronal precursors which are a precise match to the recipients own tissue and might therefore be more suitable for grafting.

Moreover the use of nuclear transfer to yield ES cells from individuals with specific genetic predispostions to certain diseases of the CNS could provide a powerful tool for the generation of in vitro models for disease pathogenesis.

It is quite likely that neural precursors generated from ES cell cultures may demonstrate a greater growth or developmental potential than committed progenitors from fetal or adult CNS.

There are a huge range of cell types within the adult CNS, and while it is clear that ES cells can give rise to any of these in the mouse, it is not clear that neural stem cells can do so.

ES derived neural progenitors may allow the study of early stages of the process of neurogenesis, and thereby provide important clues for discovery of novel factors enhancing tissue regeneration, or novel stem cell intermediates which might be more facile at replacing damaged tissue.

It may be that the frequency of homologous recombination in ES cells is much higher than that in neural stem cells, and therefore that the only practical route for introducing targetted genetic modifications into human neural tissue-either for generation of disease models in vitro or for types of gene therapy-lies in the reproducible generation and isolation of neural progenitors from genetically modified embryonic stem cells.

The present invention will now be more fully described with reference to the following examples. It should be understood, however, that the description following is illustrative only and should not be taken in any way as a restriction on the generality of the invention described above.

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EXPERIMENTAL PROTOCOLS

1. Derivation and propagation of ES cells.

Fertilised oocytes were cultured to the blastocyst stage (day 6 after insemination), in sequential media, according to a standard co-culture free protocol (Fong C.Y., and Bongso A. Comparison of human blastulation rates and total cell number in sequential culture media with and without co-culture. Hum. Reprod. 14,774-781(1999)). After zona pellucida digestion by pronase (Sigma, St. Louis,MO)(Fong C.Y. et al. Ongoing pregnancy after transfer of zona-free blastocysts: implications for embryo transfer in the human. Hum. Reprod. 12, 557-560 (1997)), ICM were isolated by immunosurgery (Solter D., and Knowles, B. Immunosurgery of mouse blastocyst. Proc. Natl. Acad. Sci. U.S.A. 72, 5099-5102 (1975)) using anti-human serum antibody (Sigma) followed by exposure to guinea pig complement (Life Technologies, Gaithersburg, MD). ICM were then

cultured on mitomycin C mitotically inactivated mouse embryonic fibroblast feeder layer (75,000 cells/cm2) in gelatine coated tissue culture dishes. The culture medium consisted of DMEM (Gibco, without sodium pyruvate, glucose 4500mg/L) supplemented with 20% fetal bovine serum (Hyclone, Logan, Utah), 0.1mM beta-mercaptoethanol, 1% non essential amino acids, 2mM glutamine, 50u/ml penicillin and 50(g/ml streptomycin (Life Technologies). During the isolation and early stages of ES cell cultivation, the medium was supplemented with human recombinant leukemia inhibitory factor hLIF at 2000u/ml (Amrad, Melbourne, Australia). 6-8 days after initial plating, ICM like clumps were removed mechanically by a micropipette from differentiated cell outgrowths and replated on fresh feeder layer. The resulting colonies were further propagated in clumps of about 100 stem cell like cells, on mouse feeder layer, about every 7 days. The clumps were either dissociated mechanically, or with a combined approach of mechanical slicing followed by exposure to dispase (10mg/ml, Life Technologies).

(a) Embryo culture

Following insemination, embryos were cultured in droplets under pre-equilibrated sterile mineral oil in IVF-50 medium (Scandinavian 2 medium) for 2 days.

A mixture 1:1 of IVF-50 and Scandinavian 2 medium (Scandinavian 2 medium) was used in the third day.

From the forth day of culture, only Scandinavian 2 medium was used to grow the cleavage stage embryos to blastocysts.

(b) Zona pellucida digestion.

Zona pellucida digestion was performed at the expanded blastocyst stage on day 6.

The digestion solution included Pronase (Sigma, TC tested) 10u in PBS and Scandinavian 2 medium (1:1).

The embryos were incubated in pronase solution for 1-1.5 min , washed in Scandinavian 2 medium and incubated for 30 minutes. If the zona was not completely dissolved, the embryos were further incubated in pronase solution for 15 seconds.

(c) .Human stem cell culture.

Human stem cells were grown on MMC treated fibroblasts' feeder layer. Fibroblasts were plated on gelatine treated dishes. A combination of human and mouse derived fibroblasts were used at a density of approximately 25,000 and 70,000 cells per cm2 respectively. The fibroblasts were plated up to 48 hours before culture of the stem cells. Mouse fibroblasts only could also support the growth of the stem cells. However, while human fibroblasts could also support stem cells, they created an uneven and unstable feeder layer. Therefore, the human fibroblasts were combined with the mouse fibroblasts to augment and achieve better support of growth and prevention of differentiation.

The medium that was used for the growth of human stem was DMEM (GIBCO, without sodium pyruvate, with glucose 4500mg/L) supplemented with 20% FBS (Hyclone, Utah) (-mercaptoethanol - 0.1mM (GIBCO), Non Essential Amino Acids - NEAA 1% (GIBCO), glutamine 2mM.(GIBCO), penicillin 50u/ml, and streptomycin 50(g/ml (GIBCO). At the initial isolation of the stem cells the medium was supplemented by hLIF 2000u/ml. It was later shown that LIF was not necessary.

(d) Human stem cell propagation:

Following plating, the isolated ICM attached and was cultured for 6 days. At that stage, a colony which included a clump of stem cells on top of differentiated cells developed. The ICM clump was isolated and removed mechanically by a micro-pipette with the aid of using Ca/Mg free PBS medium to reduce cell to cell attachments.

The isolated clump was replated on fresh human/mouse fibroblast feeder layer. Following 2 weeks of culture, a colony with typical morphology of primate pluripotent stem cells developed. The stem cells were further propagated in one of two methods. In both methods cells which appeared nondifferentiated were propagated in clumps of about 100 cells every 5-7 days.

In the first method, $Ca^{2}+/Mg^{2}+$ free PBS medium was used to reduce cell to cell attachments. Following about 15-20 minutes, cells gradually start to dissociate and the desired size clumps can be isolated. When cell dissociation is partial, mechanical dissociation using the sharp edge of the pipette assisted with cutting and the isolation of the clumps.

An alternative approach was performed by the combined use of mechanical cutting of the colonies followed by isolation of the subcolonies by dispase. Cutting of the colonies was performed in PBS containing Ca and Mg. The sharp edge of micropipette was used to cut the colonies to clumps of about 100 cells. The pipette was also used to scrape and remove differentiated areas of the colonies. The PBS was then changed to regular prequilibrated human stem cells medium containing dispase (Gibco) 10mg/ml and incubated for 5-10 minutes (at 37(C, 5% CO2). As soon as the clumps were detached they were picked up by wide bore

micro-pipette, washed in PBS containing Ca and Mg and transferred to a fresh feeder layer.

e) Human stem cell cryopreservation.

Early passage cells were cryo-preserved in clumps of about 100 cells by using the open pulled straw (OPS) vitrification method (Vajta et al 1998) with some modifications. French mini-straws (250(l, IMV, L'Aigle, France) were heat-softened over a hot plate, and pulled manually until the inner diameter was reduced to about half of the original diameter. The straws were allowed to cool to room temperature and were than cut at the narrowest point with a razor blade. The straws were sterilised by gamma irradiation (15-25 K Gy). Two vitrification solutions (VS) were used. Both were based on a holding medium (HM) which included DMEM containing HEPES buffer (Gibco, without sodium pyruvate, glucose 4500mg/L) supplemented with 20% fetal bovine serum (Hyclone, Logan, Utah). The first VS (VS1) included 10% dimethyl sulfoxide (DMSO, Sigma) and 10% ethylene glycol (EG, Sigma). The second vitrification solution (VS2) included 20% DMSO, 20% EG and 0.5M sucrose. All procedures were performed on a heating stage at 37(C. 4-6 clumps of ES cells were first incubated in VS1 for 1 minute followed by incubation in VS2 for 25 seconds. They were then washed in a 20(l droplet of VS2 and placed within a droplet of 1-2(l of VS2. The clumps were loaded into the narrow end of the straw from the droplet by capillary action. The narrow end was immediately submerged into liquid nitrogen. Straws were stored in liquid nitrogen. Thawing was also performed on a heating stage at 37(C as previously described with slight modifications (Vatja et al 1998). Three seconds after removal from liquid nitrogen, the narrow end of the straw was submerged into HM supplemented with 0.2M sucrose. After 1 minute incubation the clumps were further incubated 5 minutes in HM with 0.1M sucrose and an additional 5 minutes in HM.

2. St m cell characterisation.

Colonies were fixed in the culture dishes by 100% ethanol for immuno-fluorescence demonstration of the stem cell surface markers GCTM-2, TRA 1-60 and SSEA-1, while 90% acetone fixation was used for SSEA-4. The sources of the monoclonal antibodies used for the detection of the markers were as follows: GCTM-2, this laboratory; TRA 1-60, a gift of Peter Andrews, University of Sheffield; SSEA-1 (MC-480) and SSEA-4 (MC-813-70), Developmental Studies Hybridoma Bank, Iowa, IA. Antibody localisation was performed by using rabbit anti-mouse immunoglobulins conjugated to fluorescein isothiocyanate (Dako,Carpinteria, CA).

Alkaline phosphatase activity was demonstrated as previously described (Buehr M. and Mclaren A. Isolation and culture of primordial germ cells. Methods Enzymol. 225, 58-76, (1993)). Standard G-banding techniques were used for karyotyping.

3. Oct-4 expression studies.

To monitor expression of Oct-4, RT-PCR was carried out on colonies consisting predominantly of stem cells, or colonies which had undergone spontaneous differentiation as described below. mRNA was isolated on magnetic beads (Dynal AS, Oslo) following cell lysis according to the manufacturer's instructions, and solid-phase first strand cDNA synthesis was performed using Superscript II reverse transcriptase (Life Technologies). OCT-4 transcripts were assayed using the following primers: 5'-CGTTCTCTTTGGAAAGGTGTTC (forward) and 3'-ACACTCGGACCACGTCTTTC (reverse). As a control for mRNA quality, beta-actin transcripts were assayed using the same RT-PCR and the following primers: 5'-CGCACCACTGGCATTGTCAT-3' (forward), 5'-

TTCTCCTTGATGTCACGCAC-3' (reverse). Products were analysed on a 1.5% agarose gel and visualised by ethidium bromide staining.

4. In-vitro differentiation.

Colonies were cultured on mitotically inactivated mouse embryonic fibroblasts to confluency (about 3 weeks) and further on up to 7 weeks after passage. The medium was replaced every day. Alphafetoprotein and beta human chorionic gonadotropin levels were measured in medium conditioned by HES-1 and HES-2 at passage level 17 and 6 respectively. After 4-5 weeks of culture, conditioned medium was harvested 36 hours after last medium change, and the protein levels were determined by a specific immunoenzymometric assays (Eurogenetics, Tessenderllo, Belgium) and a fluorometric enzyme immunoassay (Dade, Miami, FL) respectively. These compounds were not detected in control medium conditioned only by feeder layer.

Differentiated cultures were fixed 6-7 weeks after passage (26 – HES-1 and 9 – HES-2) for immunofluorescence detection of lineage specific markers. After fixation with 100% ethanol, specific monoclonal antibodies were used to detect the 68 kDa neurofilament protein (Amersham, Amersham U.K), and neural cell adhesion molecule (Dako). Muscle specific actin and desmin were also detected by monoclonal antibodies (Dako) after fixation with methanol/acetone (1:1). Antibody localisation was performed as described above.

5. Teratoma formation in Severe Combined Immunodeficient (SCID) mice.

At the time of routine passage, clumps of about 200 cells with an undifferentiated morphology were harvested as described above, and

injected into the testis of 4-8 week old SCID mice (CB17 strain from the Walter and Eliza Hall Institute, Melbourne, Australia, 10-15 clumps/testis). 6-7 weeks later, the resulting tumours were fixed in neutral buffered formalin 10%, embedded in paraffin and examined histologically after hematoxylin and eosin staining.

6. Derivation and culture of neural progenitors.

Two approaches were developed for the derivation of neural precursors from human ES cells:

(a) Derivation of neural precursors from differentiating ES cells:

Colonies of undifferentiated ES cells were continuously cultured on mouse embryonic fibroblasts for 2-3 weeks. The medium was changed every day. Starting from the second week of culture and more commonly at the third week, area of tight small differentiated ES cells could be identified in the colonies both by phase contrast microscopy as well as stereo microscopy. These areas tended to become well demarcated in the third week of culture. Clumps of cells were dissected mechanically by a micropipette from these areas and were transferred to plastic tissue culture dishes containing fresh serum free medium. The medium consisted of DMEM/F12 (Gibco, Gaithersburg, MD), B27 supplementation (1:50, Gibco), glutamine 2mM (Gibco), penicillin 50u/ml and streptomycin 50μg/ml (Gibco), epidermal growth factor 20ng/ml (EGF, Gibco), and basic fibroblast growth factor 20ng/ml (bFGF, Gibco). The medium was supplemented with heparin 5μg/ml (Sigma St. Louis, MO) in some of the experiments. The clusters of cells turned into round spheres that were comprised of small tight cells within 24 hours after transfer. The spheres were sub-cultured about every 7-10 days. The diameter of spheres that were sub-cultured was usually above 0.5mm. Each sphere was dissected according to its size to 2-8 parts by two surgical blades (size 20) to

produce clumps with a maximal diameter between 0.3-0.5mm . 50% of the medium was changed about every 3 days.

(b) Derivation of neural precursors from undifferentiated ES cells:
Colonies of undifferentiated ES cells were propagated on mouse embryonic fibroblasts as described above. Undifferentiated ES cells were passaged in clumps of about 150-200 cells every 7 days. At the time of routine passage, clumps of about 200 ES cells were transferred to plastic tissue culture dishes containing the same serum free medium that was described in item 1 above. The clusters of cells turned into round spheres within 24 hours after transfer. The spheres were sub-cultured about every 7 days as described above. 50% of the medium was changed about every 3 days.

(c) Characterization of the growth and the number of cells in the spheres.
Growth of the progenitors was roughly evaluated by the increase in the number of spheres at each passage. The growth was also monitored for individual spheres that were plated in twenty four well dishes (a sphere per well). The growth of the spheres was evaluated by serial measurements of their diameter every 3-4 days starting from 24-48 hours after plating.

The number of cells per sphere and its correlation with the diameter of the spheres was evaluated in a sample of spheres with various sizes. Each sphere was disaggregated into single cells by enzymatic (papain, Wortinington Biochemical Co, NJ) digestion that was followed by tituration. The cells were than spun down re-suspended in serum free medium and counted. The cells were also stained with trypan blue to determine the rate of viable cells.

7. Characterization of the progenitor cells in the spheres.

(a) Immunohistochemistry studies

The spheres were plated on coverslips coated with poly-D-lysine (30-70 kDa, Sigma) and laminin (Sigma), fixed after 4 hours and examined by indirect immunofluorescence analysis for expression of N-CAM (acetone fixation, mouse monoclonal antibody UJ13a from Dako,Carpinteria, CA), nestin (4% paraformaldehyde fixation, rabbit antiserum a kind gift of Dr. Ron McKay) and vimentin (methanol fixation, mouse monoclonal antibody Vim3B4 from Roche Diagnostics Australia, Castle Hill, NSW).

To evaluate the rate of cells that expressed NCAM, the spheres were dissaggregated by mechanical tituration in PBS without calcium and magnesium. The resulting small clumps of cells and single cells were plated on coverslips coated with poly-D-lysine and laminin fixed after 24 hours and examined by indirect immunofluorescence analysis for expression of N-CAM.

(b) RT-PCR

The expression of nestin, the transcription factor PAX-6 and (-actin in the spheres was demonstrated by RT-PCR.

mRNA was isolated on magnetic beads (Dynal AS, Oslo) following cell lysis according to the manufacturer's instructions, and solid-phase first strand cDNA synthesis was performed using Superscript II reverse transcriptase (Gibco, Gaithersburg, MD).

The PCR reaction was carried out according to van Eijk et al. (1999), using the solid phase cDNA as template and Taq polymerase (Pharmacia Biotech, Hong Kong). PCR primers were synthesized by Besatec or Pacific Oligos (Adelaide, Australia). The following primers were used for PAX-6 and nestin respectively: Pax-6 forward primer,

5'AACAGACACAGCCCTCACAAACA3'; Pax-6 reverse primer, 5'CGGGAACTTGAACTGGAACTGAC3'; nestin forward primer, 5'CAGCTGGCGCACCTCAAGATG3'; nestin reverse primer, 5'AGGGAAGTTGGGCTCAGGACTGG3'. As a control for mRNA quality, we assayed beta-actin transcripts using the same RT-PCR and the following primers:

5'-CGCACCACTGGCATTGTCAT-3' (forward), and
5'-TTCTCCTTGATGTCACGCAC-3' (reverse). Products were analysed on a 1.5% agarose gel and visualised by ethidium bromide staining.

(c) Neuronal differentiation studies

In general, differentiation was induced by plating the spheres on an appropriate substrate (poly-D-lysine, 30-70 kDa, and laminin, Sigma) combined with the removal of growth factors.

Two protocols were most commonly used: In the first one, differentiation was induced by plating the spheres on coverslips coated with poly-D-lysine and laminin in the same serum free medium detailed above without growth factors supplementation. The cells in the spheres were allowed to spread and differentiate for 2-3 weeks and the medium was changed every 3-5 days. In some of the experiments, starting from the sixth day after plating, the medium was supplemented with all trans retinoic acid (Sigma, 10-6M).

In the second protocol, the spheres were plated on coverslips coated with poly-D-lysine and laminin in serum free growth medium supplemented with growth factors. After 5-6 days the supplementation of growth factors was withdrawn and all trans retinoic acid (Sigma, 10-6M) was added to the medium. The cells were further cultured for 1-2 weeks. The medium was changed every 5 days.

(d) Characterization of differentiated neuronal cells

Differentiated cells growing out from the spheres were analysed 2-3 weeks after plating by indirect immunofluorescence for the expression of the following markers: 200 kDa neurofilament protein (4% paraformaldehyde fixation, mouse monoclonal antibody RT97 from Novocastra, Newcastle, UK), 160 kDa neurofilament protein (methanol fixation, mouse monoclonal NN18 from Roche) 68kDa neurofilament protein (100% ethanol, Amersham, Amersham U.K), MAP2 a,b (4%paraformaldehyde fixation, mouse monoclonal AP20 from Neomarkers, Union City CA), glutamate (1% paraformaldehydeand 1%glutaraldehyde, rabbit antiserum from Sigma), synaptophysin (4%paraformaldehyde, mouse monoclonal SY38 from Dako), glutamic acid decarboxylase (1% paraformaldehyde, 1 % glutaraldehyde, rabbit antiserum from Chemicon, Temecula, CA), β-tubulin (4%paraformaldehyde, mouse monoclonal TUB 2.1 from Sigma) and β-tubulin III (4%paraformaldehyde mouse monoclonal SDL.3D10 from Sigma) .

Differentiated cells were also analysed 2-3 weeks after plating by RT-PCR for the expression of (-actin, glutamic acid decarboxylase (primers, Vescovi et al 1999) and GABAA receptor subunit (2 (primers, Neelands et al 1998). mRNA preparation and the RT-PCR reaction were carried out as described above.

(e) Glial differentiation studies.

At the time of routine passage spheres were subcultured into serum free medium (as detailed above) supplemented with platelet derived growth factor (recombinant human PDGF-AA, Peprotech Inc 20ng/ml) and bFGF (Gibco, 20ng/ml). Fifty percent of the medium was replaced by fresh medium every 3 days. After culture for 6 days the spheres were plated on

coverslips coated with poly-D-lysine and laminin in the same serum free medium without growth factors supplementation. The cells in the spheres were allowed to spread and differentiate for 10-12 days and the medium was changed every 3-5 days. An alternative protocol was used in some of the experiments. In these experiments the spheres were cultured in serum free medium (as detailed above) supplemented with PDGF-AA, (20ng/ml) and bFGF (20ng/ml) for three weeks. The spheres were then plated on coverslips coated with poly-D-lysine and Fibronectin (Sigma, 5mcg/mk). They were cultured for a week in the serum free medium supplemented with PDGF-AA, (20ng/ml), bFGF (20ng/ml) and T3 (30nM). The growth factors were then removed from the medium and the cells were further cultured for another 1-2 weeks in the presence of T3 only. Fifty percent of the medium was replaced by fresh medium every 3 days. In an alternative approach, spheres that were propagated in the presence of EGF and bFGF were plated on coverslips coated with poly-D-lysine and Fibronectin (Sigma, 5mcg/mk). EGF was removed from the medium and the spheres were cultured in the presence of T3 (30nM) and bFGF (20ng/ml) for a week. The cells were then further cultured for another 3-4 weeks in the presence of T3 and PDGF-AA (20ng/ml). Oligodendrocyte were identified at that time by indirect immunofluorescence for the expression of the marker O4. The cells were first incubated with the primary (Anti oligodendrocyte marker O4, mouse monoclonal IgM from Chemicon Int. Inc. Temecula, CA) and secondary FITC or rhodamine conjugated antibodiesand were then fixed with 4% paraformaldehyde.

EXAMPLES

Example 1 - Derivation of cell lines HES-1 and HES-2

The outer trophectoderm layer was removed from four blastocysts by immunosurgery to isolate inner cell masses (ICM), which were then plated onto a feeder layer of mouse embryo fibroblasts (Figure 1A). Within several days, groups of small, tightly packed cells had begun to proliferate

from two of the four ICM. The small cells were mechanically dissociated from outgrowths of differentiated cells, and following replating they gave rise to flat colonies of cells with the morphological appearance of human EC or primate ES cells (Figure 1B, C stem cell colonies). These colonies were further propagated by mechanically disaggregation to clumps which were replated onto fresh feeder cell layers. Growth from small clumps of cells (<10 cells) was not possible under the conditions of these cultures. Spontaneous differentiation, often yielding cells with the morphological appearance of early endoderm, was frequently observed during routine passage of the cells (Figure 1D). Differentiation occurred rapidly if the cells were deprived of a feeder layer, even in the presence of LIF (Figure 1E). While LIF was used during the early phases of the establishment of the cell lines, it was subsequently found to have no effect on the growth or differentiation of established cultures (not shown). Cell line HES-1 has been grown for 60 passages in vitro and HES-2 for 40 passages, corresponding to a minimum of approximately 360 and 90240 population doublings respectively, based on the average increase in colony size during routine passage, and both cell lines still consist mainly of cells with the morphology of ES cells. Both cell lines have been successfully recovered from cryopreservation.

Example 2 - Marker expression and karyotype of the human ES cells

Marker and karyotype analysis were performed on HES-1 at passage levels 5-7, 14-18, 24-26 and 44-46, and on HES-2 at passage levels 6-8. ES cells contained alkaline phosphatase activity (Figure 2A). Immunophenotyping of the ES cells was carried out using a series of antibodies which detect cell surface carbohydrates and associated proteins found on human EC cells. The ES cells reacted positively in indirect immunofluorescence assays with antibodies against the SSEA-4 and TRA 1-60 carbohydrate epitopes, and the staining patterns were similar to those observed in human EC cells (Figure 2B, C). ES cells also

reacted with monoclonal antibody GCTM-2, which detects an epitope on the protein core of a keratan sulphate/chondroitin sulphate pericellular matrix proteoglycan found in human EC cells (Figure 2D). Like human EC cells, human ES cells did not express SSEA-1, a marker for mouse ES cells. Both cell lines were karyotypically normal and both were derived from female blastocysts.

Oct-4 is a POU domain transcription factor whose expression is limited in the mouse to pluripotent cells, and recent results show directly that zygotic expression of Oct-4 is essential for establishment of the pluripotent stem cell population of the inner cell mass. Oct-4 is also expressed in human EC cells and its expression is down regulated when these cells differentiate. Using RT-PCR to carry out mRNA analysis on isolated colonies consisting mainly of stem cells, we showed that human ES cells also express Oct-4 (Figure 3, lanes 2-4). The PCR product was cloned and sequenced and shown to be identical to human Oct-4 (not shown).

Example 3 - Differentiation of human ES cells *in vitro*

Both cell lines underwent spontaneous differentiation under standard culture conditions, but the process of spontaneous differentiation could be accelerated by suboptimal culture conditions. Cultivation to high density for extended periods (4-7 weeks) without replacement of a feeder layer promoted differentiation of human ES cells. In high density cultures, expression of the stem cell marker Oct-4 was either undetectable or strongly downregulated relative to the levels of the housekeeping gene beta actin (Figure 3, lanes 5-7). Alphafetoprotein and human chorionic gonadotrophin were readily detected by immunoassay in the supernatants of cultures grown to high density. Alphafetoprotein is a characteristic product of endoderm cells and may reflect either extraembryonic or embryonic endodermal differentiation; the levels observed (1210-5806

ng/ml) are indicative of extensive endoderm present. Human chorionic gonadotrophin secretion is characteristic of trophoblastic differentiation; the levels observed (6.4-54.6 IU/Litre) are consistent with a modest amount of differentiation along this lineage.

After prolonged cultivation at high density, multicellular aggregates or vesicular structures formed above the plane of the monolayer, and among these structures clusters of cells or single cells with elongated processes which extended out from their cell bodies, forming networks as they contacted other cells (Figure 1F) were observed. The cells and the processes stained positively with antibodies against neurofilament proteins and the neural cell adhesion molecule (Figure 2E and F). Contracting muscle was seen infrequently in the cultures. While contracting muscle was a rare finding, bundles of cells which were stained positively with antibodies directed against muscle specific forms of actin, and less commonly cells containing desmin intermediate filaments (Figure 2G and H) were often observed. In these high density cultures, there was no consistent pattern of structural organisation suggestive of the formation of embryoid bodies similar to those formed in mouse ES cell aggregates or arising sporadically in marmoset ES cell cultures.

Example 4 - Differentiation of human ES cells in xenografts

When HES-1 or HES-2 colonies of either early passage level (6; HES 1 and 2) or late passage level (HES-1, 14 and 27) were inoculated beneath the testis capsule of SCID mice, testicular lesions developed and were palpable from about 5 weeks after inoculation. All mice developed tumours, and in most cases both testis were affected. Upon autopsy lesions consisting of cystic masses filled with pale fluid and areas of solid tissue were observed. There was no gross evidence of metastatic spread to other sites within the peritoneal cavity. Histological examination

revealed that the lesion had displaced the normal testis and contained solid areas of teratoma. Embryonal carcinoma was not observed in any lesion. All teratomas contained tissue representative of all three germ layers. Differentiated tissues seen included cartilage, squamous epithelium, primitive neuroectoderm, ganglionic structures, muscle, bone, and glandular epithelium (Figure 4). Embryoid bodies were not observed in the xenografts.

Example 5 - Development, propagation and characterisation of human ES cells derived neural progenitor cells.

a) Derivation of neural progenitor cells from human ES cells.

Colonies of undifferentiated ES cells from the cell lines HES-1 and HES-2 were continuously cultured on mouse embryonic fibroblasts feeder layer for 2-3 weeks. From the second week, areas with differentiated small piled tightly packed cells could be identified in the colonies of both cell lines by phase and inverted microscope. During the third week these areas became more defined from neighboring areas of the colony. The cells in these areas were not reactive in immunohistochemical staining with the antibody against the early neuroectodermal marker NCAM. In 54% of the colonies (67/124, HES-1) the areas were large and well demarcated sufficiently to allow mechanical removal of clumps of cells by a micropipette. Clumps, which were comprised mostly of the small tightly packed cells (about 100-300cells/clump), were removed from differentiating colonies of HES-1 and HES-2 and were transferred to serum free medium supplemented with basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF). Within an hour the clumps started to change their shape toward spheres and after 24 hours all the clumps turned into round spheres (figure 5a).

After 7-10 days in culture, gradual increase in the size of the majority of the spheres was evident and most of the spheres were still floating or loosely attached to the dish while a minority attached and started to spread.

In an alternative approach, somatic differentiation of ES cells into spheres of progenitor cells was induced by transferring clumps of undifferentiated ES cells into serum free medium supplemented with basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF). Within 24 hours the clumps have turned into spheres. Some of these spheres were round and some had an irregular shape. After 72 hours in serum free medium 42% (10/24) of the spheres had a round symmetrical appearance (Figure 9) and after 12 days 62.5% (15/24). Significant growth was observed in the majority of the spheres during this early culture. It was possible to measure and calculate the average volume of the round floating spheres and it increased by 64% (mean growth of 15 spheres) between days 5 and 12.

b) In vitro propagation of spheres of progenitor cells

After 7-10 days in culture, floating or loosely attached spheres with a diameter of > 0.5mm were sub-cultured by mechanical dissection into 2-8 pieces, which were re-plated in fresh pre-equilibrated medium. The spheres were cultivated in this manner during a five months period (15 passages). Although some of the spheres had an irregular shape at the initial phase of culture, the rate of round symmetrical spheres increased along propagation., In addition, while at early passage levels the appearance (under a stereo-microscope) of the inner part of the spheres was irregular, it gradually turned to be uniform at more advanced passage levels. By passage level ten all spheres had a round symmetrical shape and a uniform appearance.

Proliferation of the cells was evaluated by determining the increase in the number of spheres with each passage as well as measuring the increase in the volume of the spheres along time . The number of spheres increased by 126% + 54% (Mean+SD, sum of results from 3 cell lines) at each passage (performed every 7 days) during the first 5 passages. A gradual decline in the growth of the number of spheres with each passage was evident during the next 5 passages reaching an increase of 28% (mean data from 2 cell lines) at passage level 10. The mean volume of the spheres also increased by similar rates. A mean increase of 22.3%+ 24.3% (data from 24 spheres) per week was evident at passage level 10. A stable growth of the spheres was evident from passage level 10 and on being 21.8+12.3%, 42.1+20%, 18.4+17.8% and 38.7+28.7% (Mean+SD% of 6 spheres) per each consecutive week after passage 10.

Attempts to dis-aggregate the sphere by using trypsin digestion were not successful, however it was possible to dis-aggregate them into a single cell suspension mechanically following enzymatic digestion with papain. . A linear correlation was found between the volume of spheres and the number of cells within the spheres (figure 10).

Given the growth rate of the spheres with each passage and the number of cells (20,000, figure 10) per averaged size sphere (0.1mm^3 based on the mean diameter + S.D. of 24 spheres 7 days after passage 5, 0.59 + 0.14mm), it was calculated that 10 clumps of ES cells may generate within 10 passages 2500 spheres containing 50 x 10^6 cells.

Spheres that were cultured in the serum free growth medium (supplemented with growth factors) for prolonged periods (2-3 weeks) without passage, have attached to the tissue culture plastic and gradually spreaded as a monolayer of cells. The cells in the monolayer had a

uniform appearance of neural progenitors and a high mitotic activity was evident (Figure 7).

c) Characterization of the progenitor cells.

Cells in the spheres that were produced either from differentiating ES cell colonies or directly from undifferentiated ES cells expressed markers of primitive neuroectoderm, such as polysialylated N-CAM (Figure 5b, 11,12), the intermediate filament proteins nestin (immunostaining, Figure 5cand 13; RT-PCR, figure 3b) and vimentin (Figure 5d and 15), and the transcription factor Pax-6 (Figure 3b).

To evaluate the percentage of cells in the spheres that expressed NCAM, spheres at an advanced passage level were disaggregated by mechanical trituration in PBS without calcium and magnesium. The resulting single cells or small clumps of cells were plated on substrate in growth medium. Twenty four hours after plating, 98.3% of the cells were decorated with the antibody against NCAM.

The percentage of cells in the spheres at an advanced passage level that could express nestin was determined in cells that were spreading in a monolayer from spheres, after prolonged culture in the presence of growth factors. These cells uniformaly expressed the intermediate filament nestin.

These data indicate that the spheres were comprised of a purified preparation of progenitor cells capable of expressing nestin and NCAM.

(d) *In Vitro* neuronal Differentiation

When plated on an appropriate substrate, the spheres attached, and differentiated cells grew out onto the monolayer from them.

When the bFGF and EGF were removed at the time of plating, differentiating cells gradually spread from them in a radial fashion (Figure 6a) If the growth factors were removed only after 6 days, a more extensive spreading of cells with processes, which formed a monolayer was evident (Figure 8). Two to three weeks after plating, the differentiated cells displayed morphology and expression of structural markers characteristic of neurons, such as β-tubulin, the 200kDa neurofilament and 68kDA neurofilament proteins (Figure 6b and h). Moreover, the differentiated cells expressed markers of mature neurons such as the 160kDa neurofilament protein (Figure 6c), Map-2a,b (Figure 6d) and synaptophysin (Figure 6F),Furthermore, the cultures contained cells which synthesised glutamate (Figure 6e), expressed the rate limiting enzyme in GABA biosynthesis) glutamic acid decarboxylase, Figure 3c and 6g) and receptor subunits characteristic of GABAminergic neurons (GABAa2, Figure 3d).

e) *In vitro* glial differentiation

To promote differentiation towards the glial lineage, spheres were initially cultured for 6 days in serum free medium supplemented with PDGF (20ng/ml) and bFGF (20ng/ml) and were then plated on coverslips coated with poly-D-lysine and laminin in the same medium without growth factors supplementation. The cells in the spheres were allowed to spread and differentiate for 10-12 days. Small cells decorated with the antibody O4 could be demonstrated at that time indicating differentiation into oligodendrocyte progenitors.

It was also possible to promote the differentiation into oligodendrocyte progenitors by incubation of the spheres in the presence of PDGF and basic FGF for 3 weeks followed by plating on poly lysine and fibronectin and culture for a week in the presence of the growth factors and T3 followed by 1-2 weeks culture in the presence of T3 without growth factors supplementation (figure 14). Alternatively, spheres that were propagated

in the presence of bFGF and EGF were plated on poly lysine and fibronectin and cultured for a week in the presence of bFGF and T3. The cells were then further cultured in the presence of PDGF and T3 for 3-4 weeks.

Example 6: Cryo-preservation of human ES cells.

Attempts to cryo-preserve human ES cells by using conventional slow freezing protocols were associated with a very poor outcome after thawing. Since ES cells are derived from the blastocyst and retain their embryonic properties in culture, we have postulated that cryopreservation by using a method which is efficient for embryos may be beneficial. Early passage clumps of human ES cells were frozen by using the open pulled straw (OPS) vitrification method which was recently shown to be highly efficient for the cryopreservation of bovine blastocysts (Vatja et al. 1998). Both cell lines were successfully thawed and further propagated for prolonged periods. The outcome of the vitrification procedure was further studied on cell line HES-1, and recovery of viable cells with this procedure was found to be highly efficient. All clumps (n=25) survived the procedure and attached and grew after thawing. Vitrification was associated with some cell death as evidenced by the reduced size of colonies originating from vitrified clumps two days after thawing in comparison to colonies from non-vitrified control clumps. However, two days in culture were sufficient to overcome this cell deficit, and 9 days after plating the size of colonies from frozen-thawed clumps exceeded that of control colonies at 7 days. Vitrification did not induce differentiation after thawing. Thawed cells retained a normal karyotype and the expression of primate stem cell markers, and formed teratomas in SCID mice.

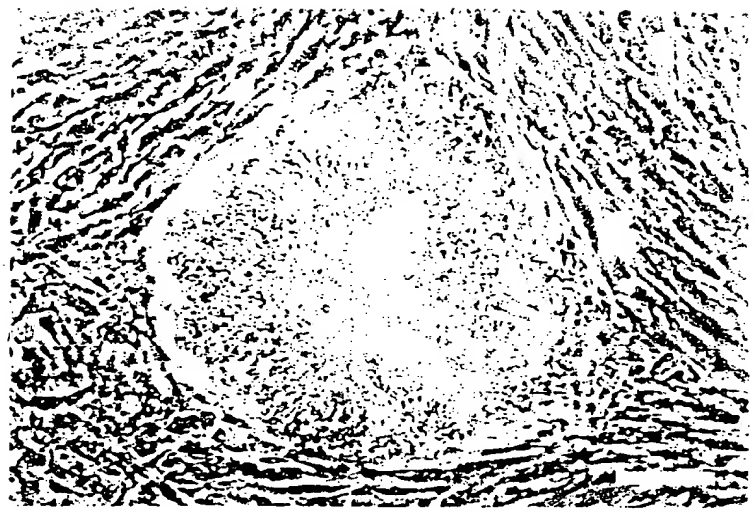
Finally it is to be understood that various other modifications and/or alterations may be made without departing from the spirit of the present invention as outlined herein.

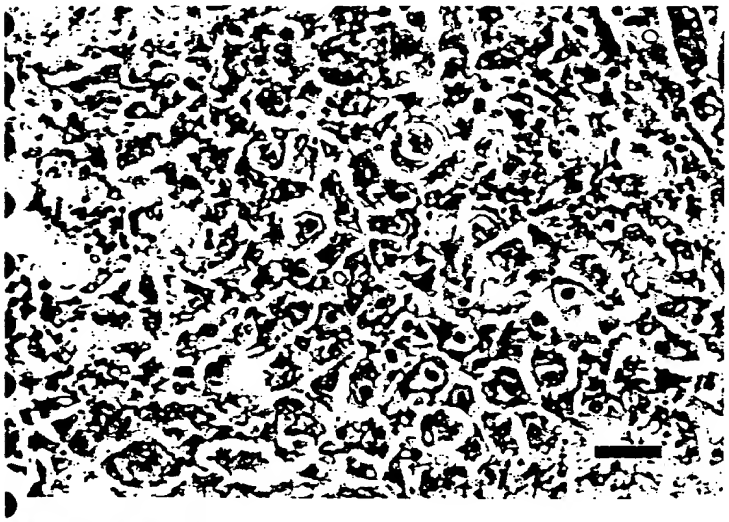
DATED: 6 November, 2000

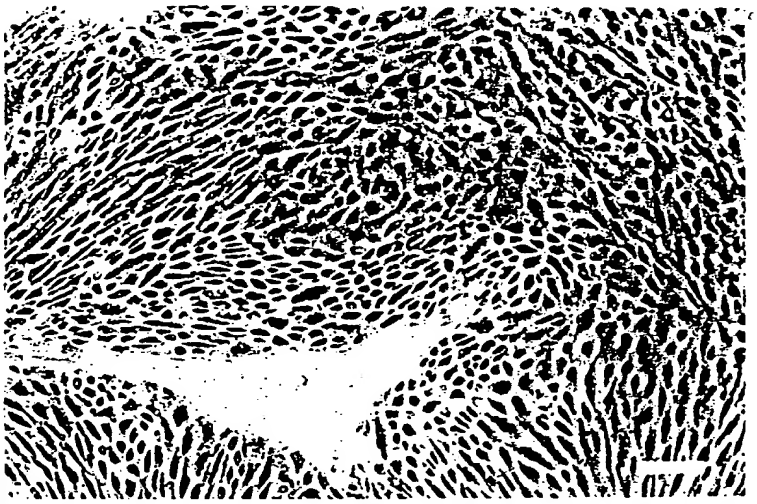
PHILLIPS ORMONDE & FITZPATRICK
Attorneys for:
MONASH UNIVERSITY, NATIONAL UNIVERSITY OF SINGAPORE and HADASIT MEDICAL RESEARCH SERVICES & DEVELOPMENT CO LTD

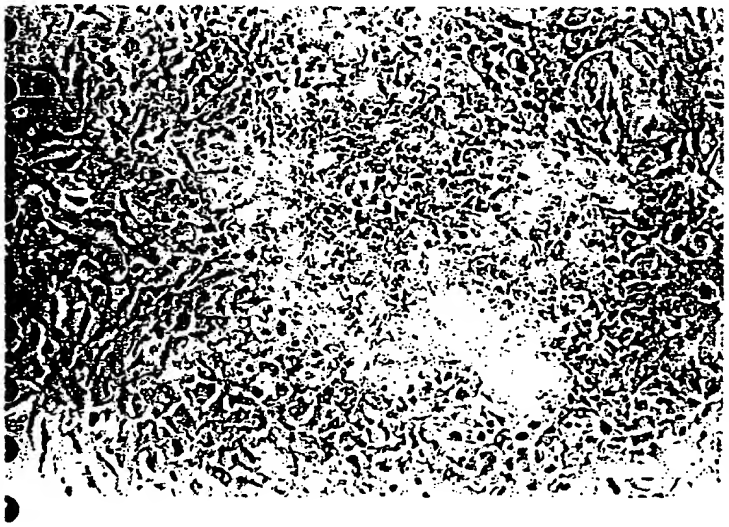
David B Fitzpatrick

Figure 1 ab
cd
ef









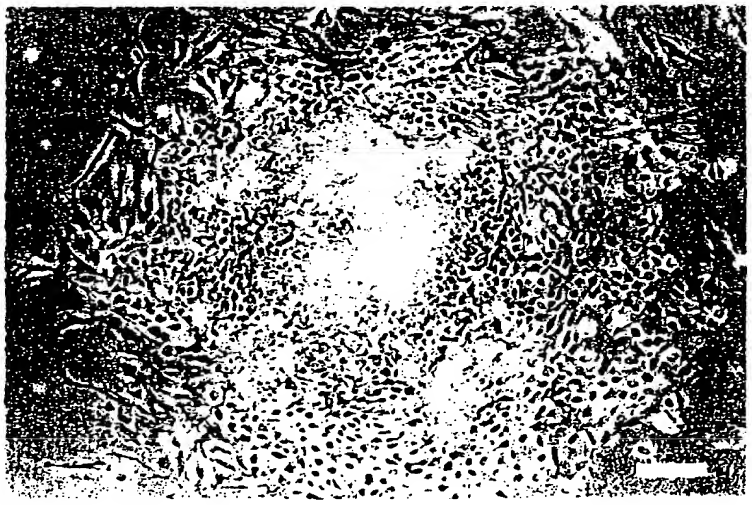


Figure 2 ab
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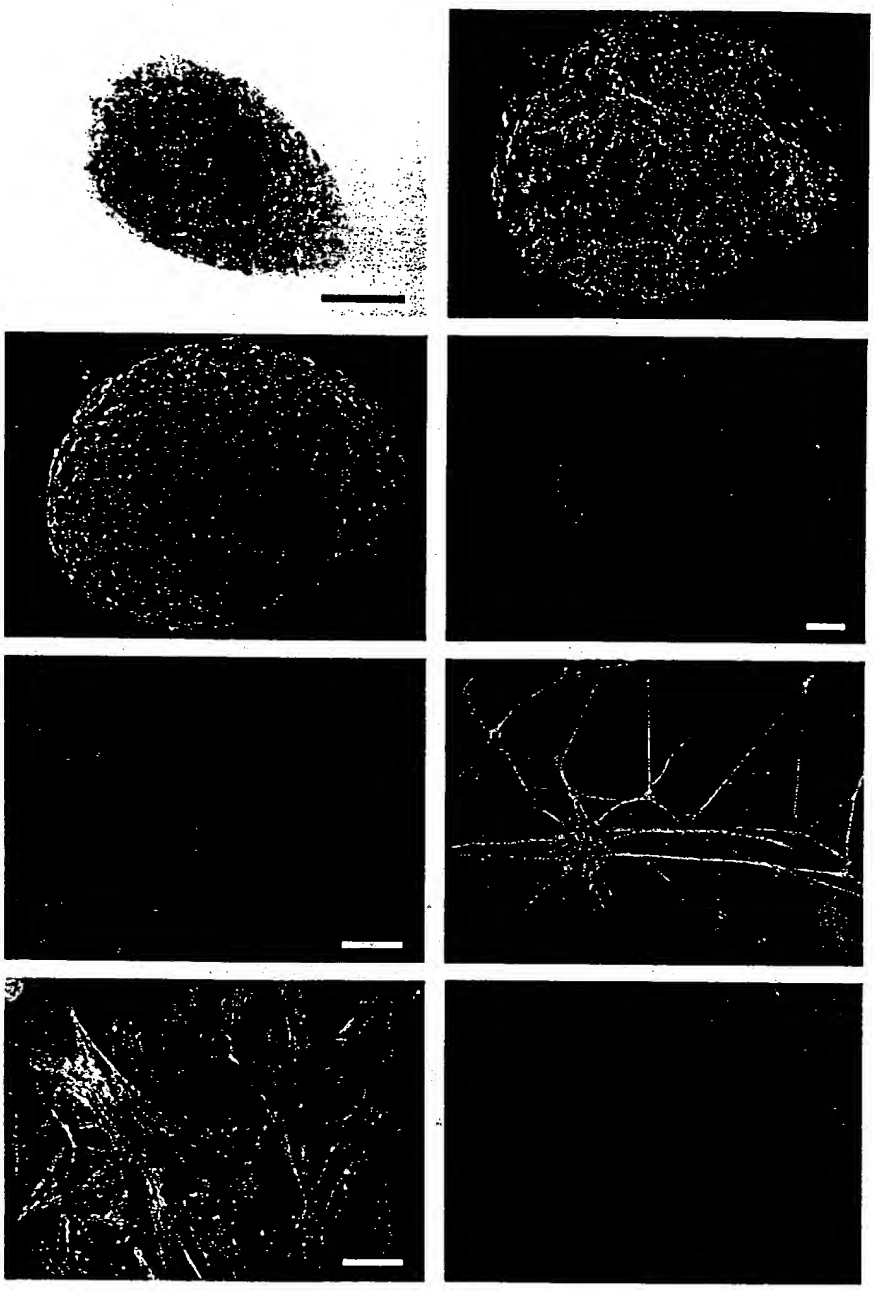
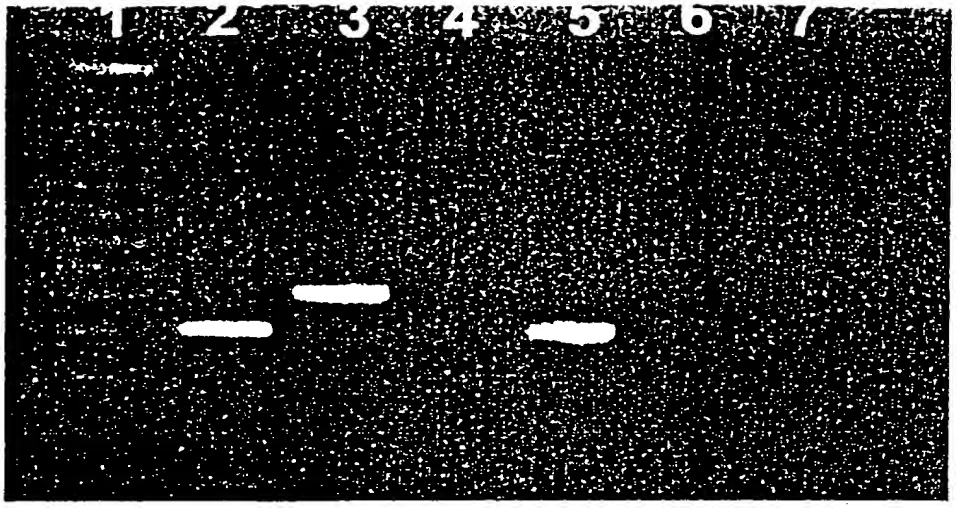
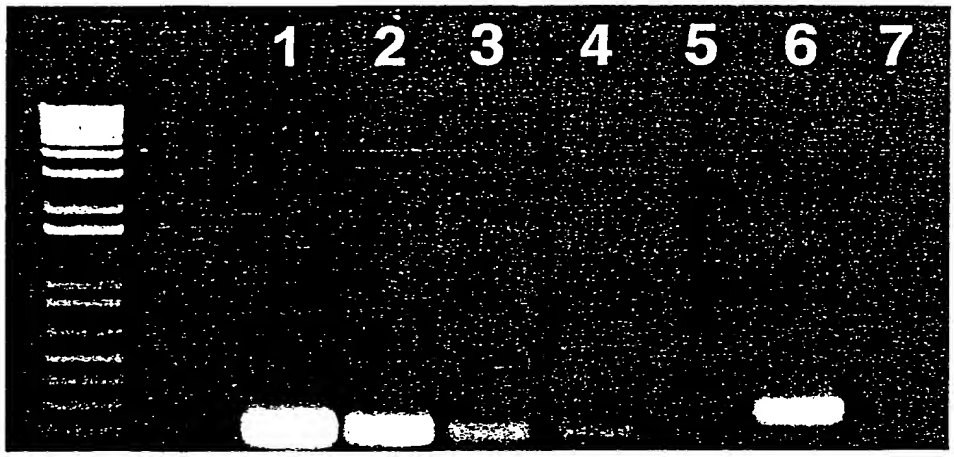
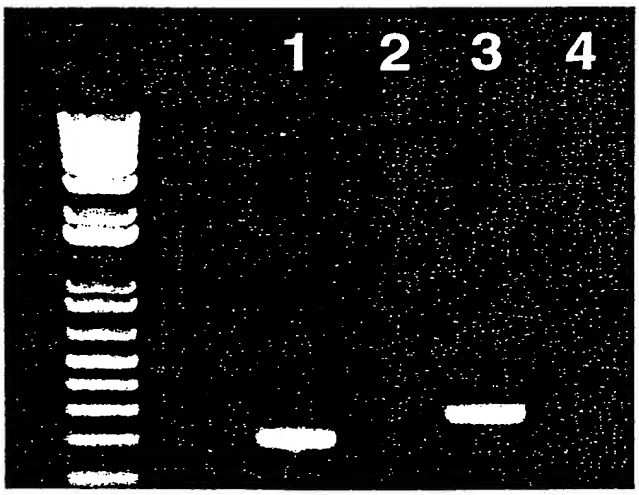


Figure 3 a
b
c
d







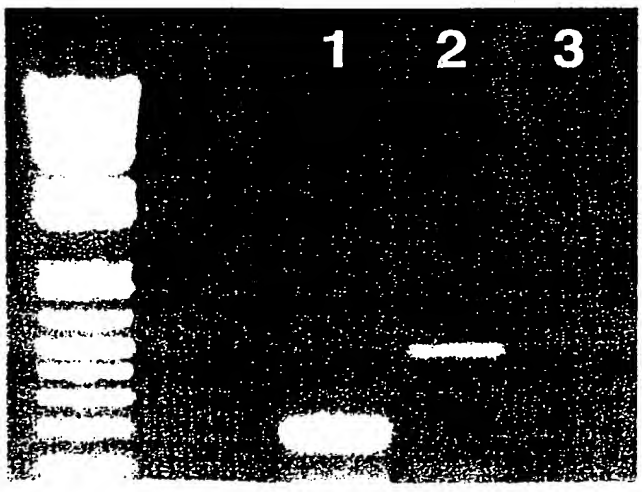


Figure 4 ab
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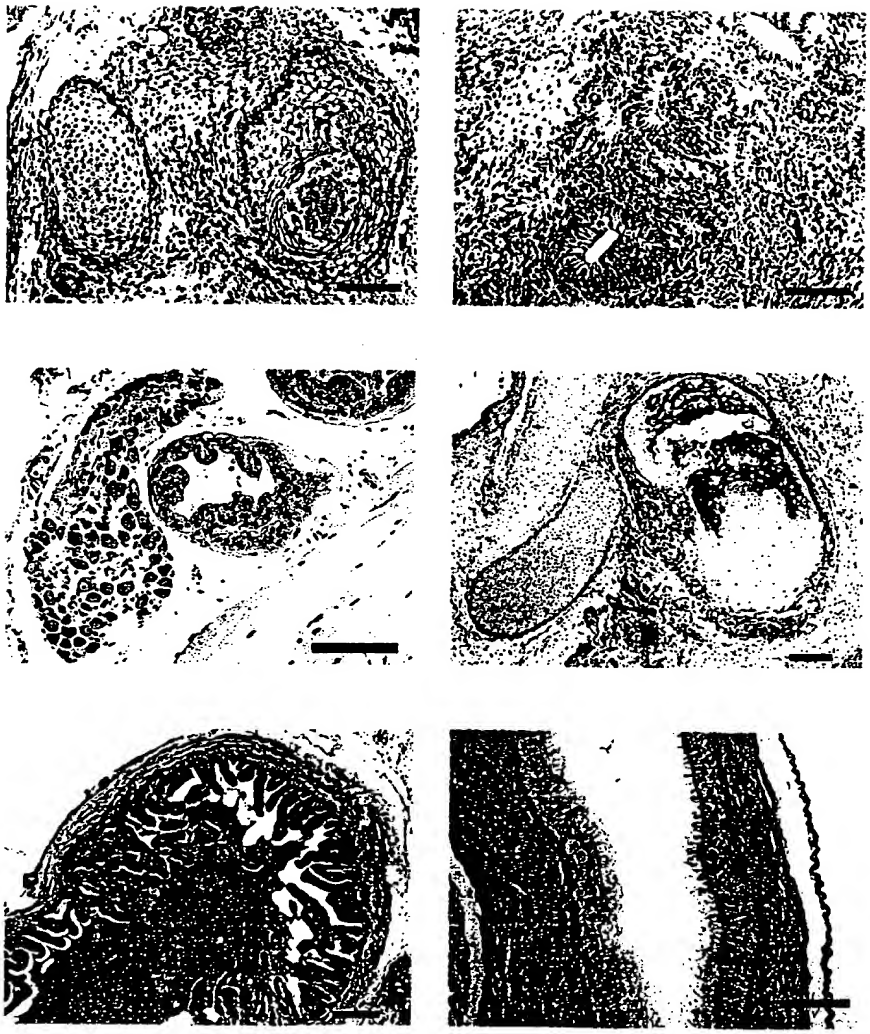

Figure 5 ab
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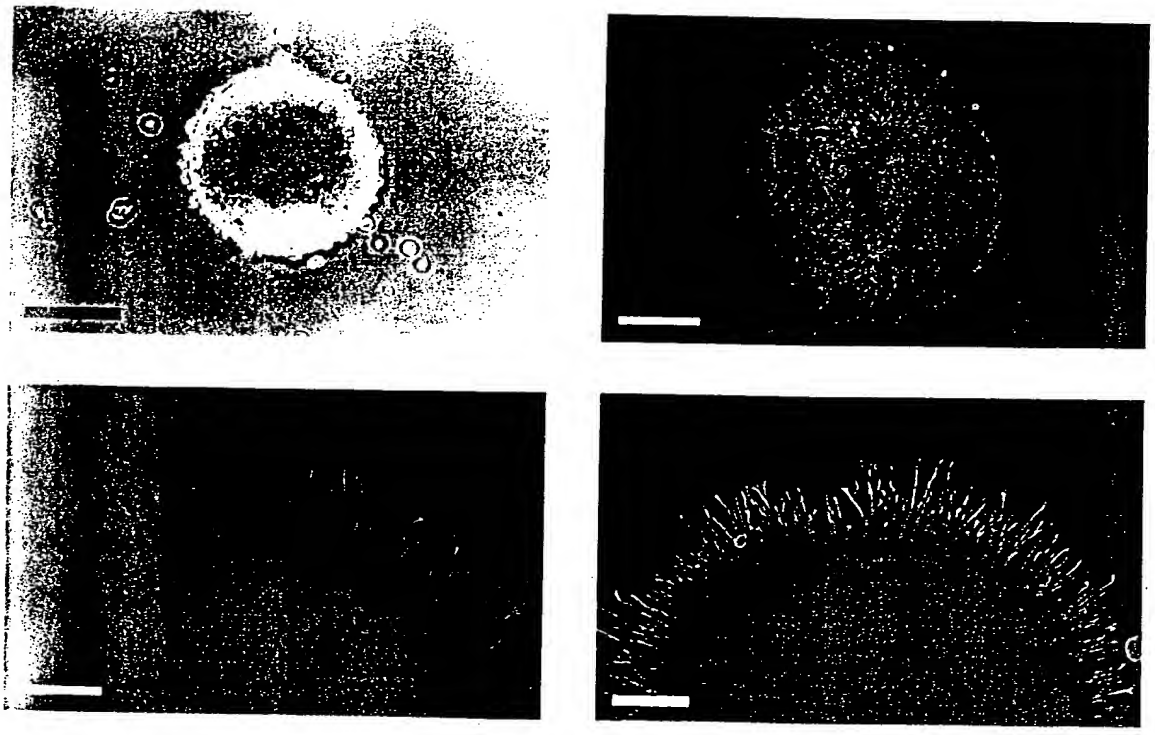

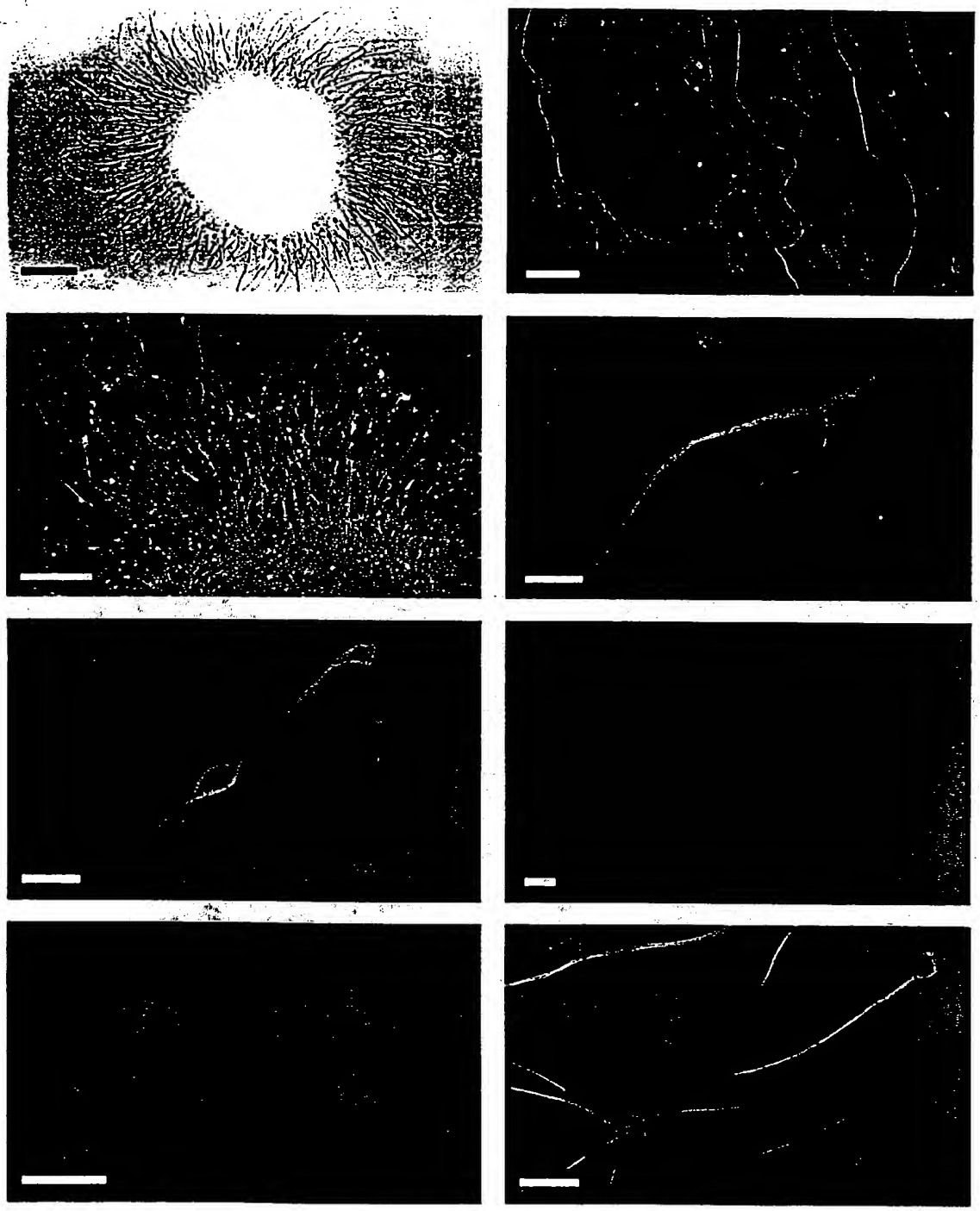

Figure 6 ab
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Figure 7

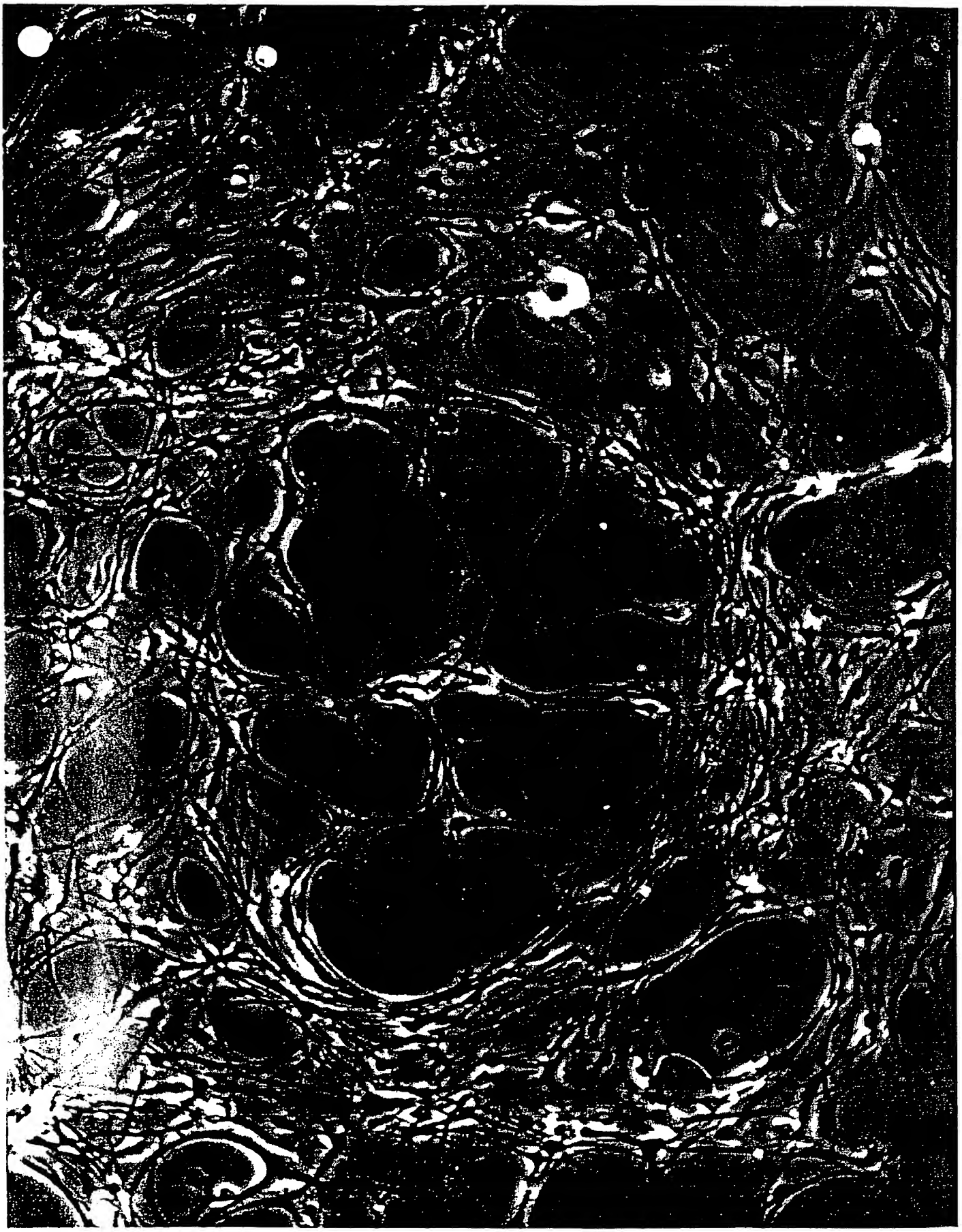


FIGURE 8

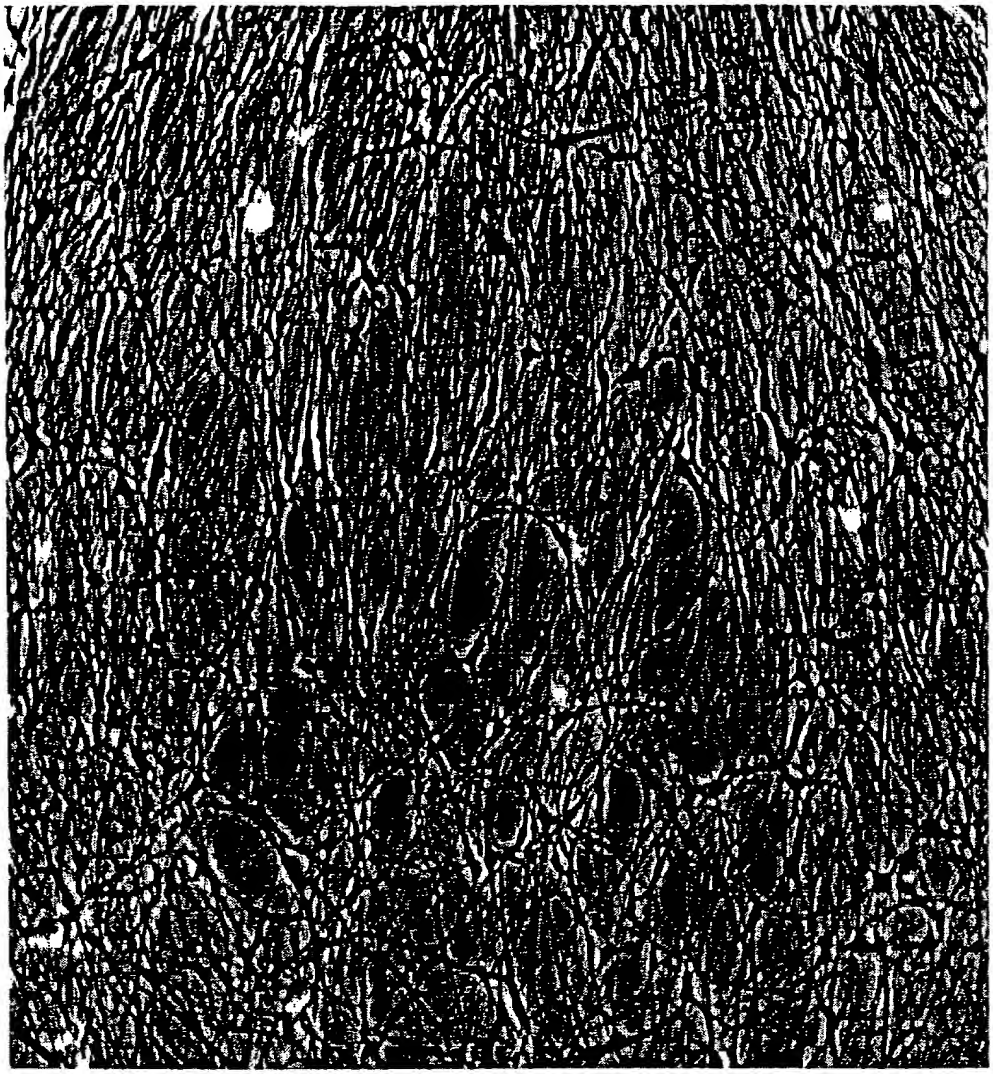


Figure 9:

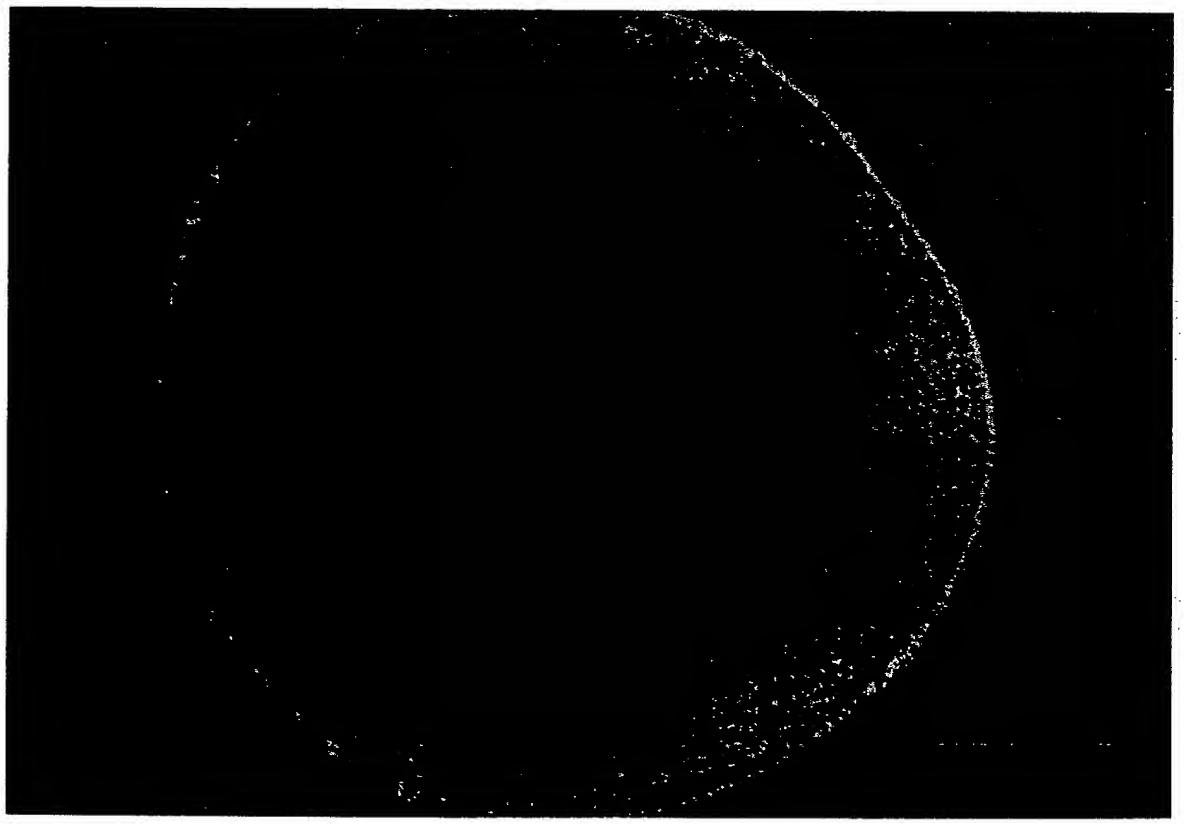


FIGURE 10

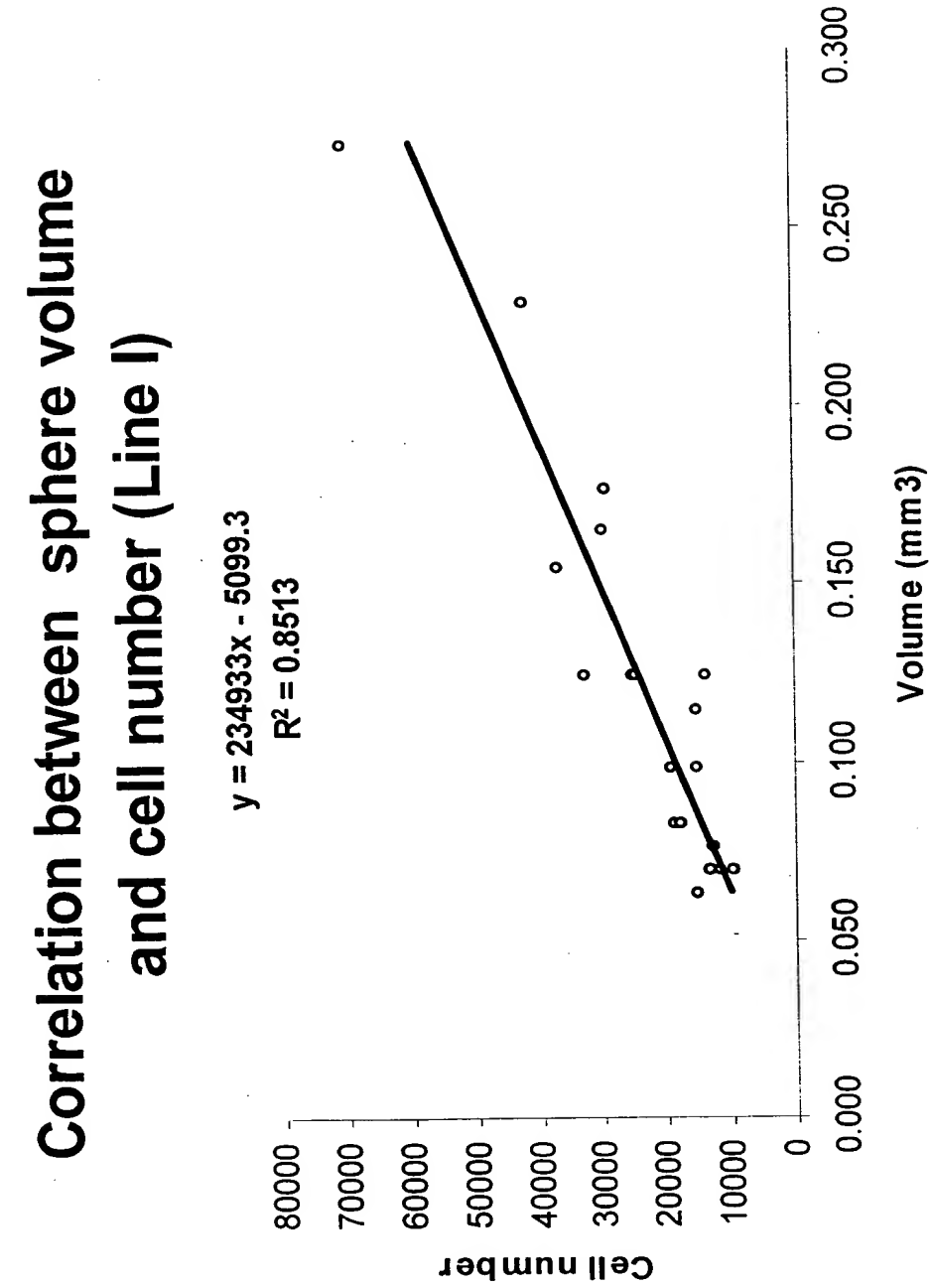

Correlation between sphere volume and cell number (Line I)
y = 234933x - 5099.3
R² = 0.8513
Cell number
80000
70000
60000
50000
40000
30000
20000
10000
0
0.000
0.050
0.100
0.150
0.200
0.250
0.300
Volume (mm3)

Figure 11:

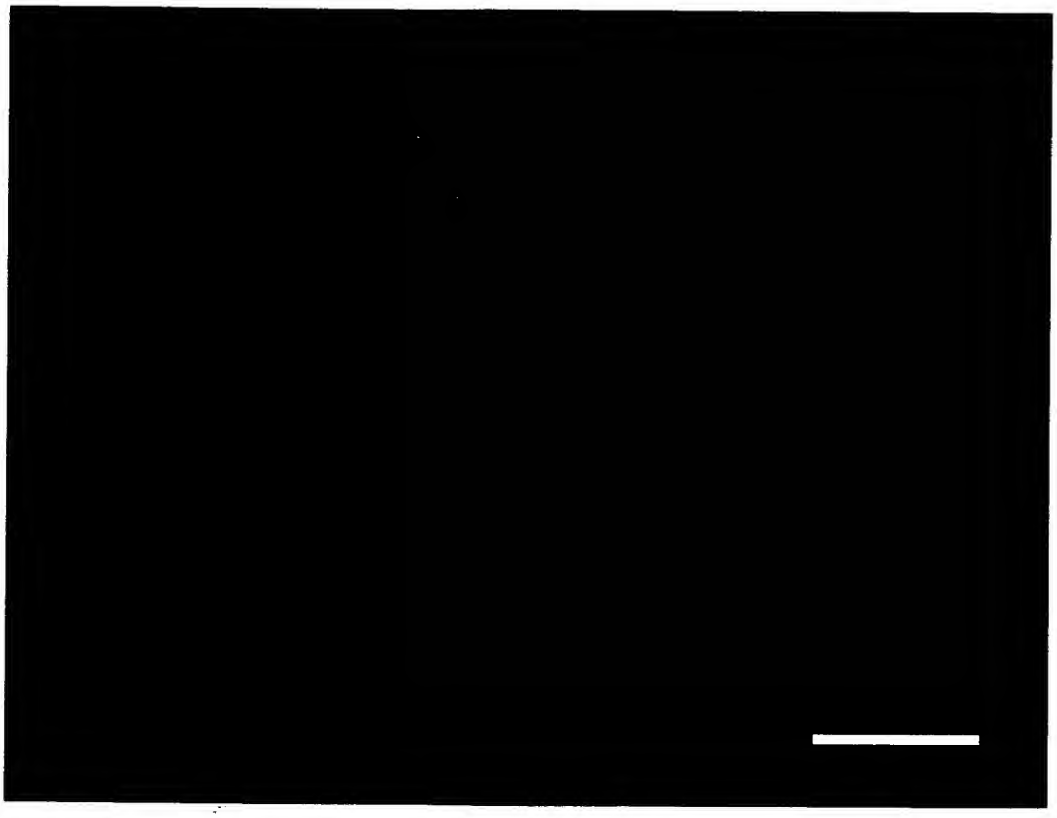


Figure 12:

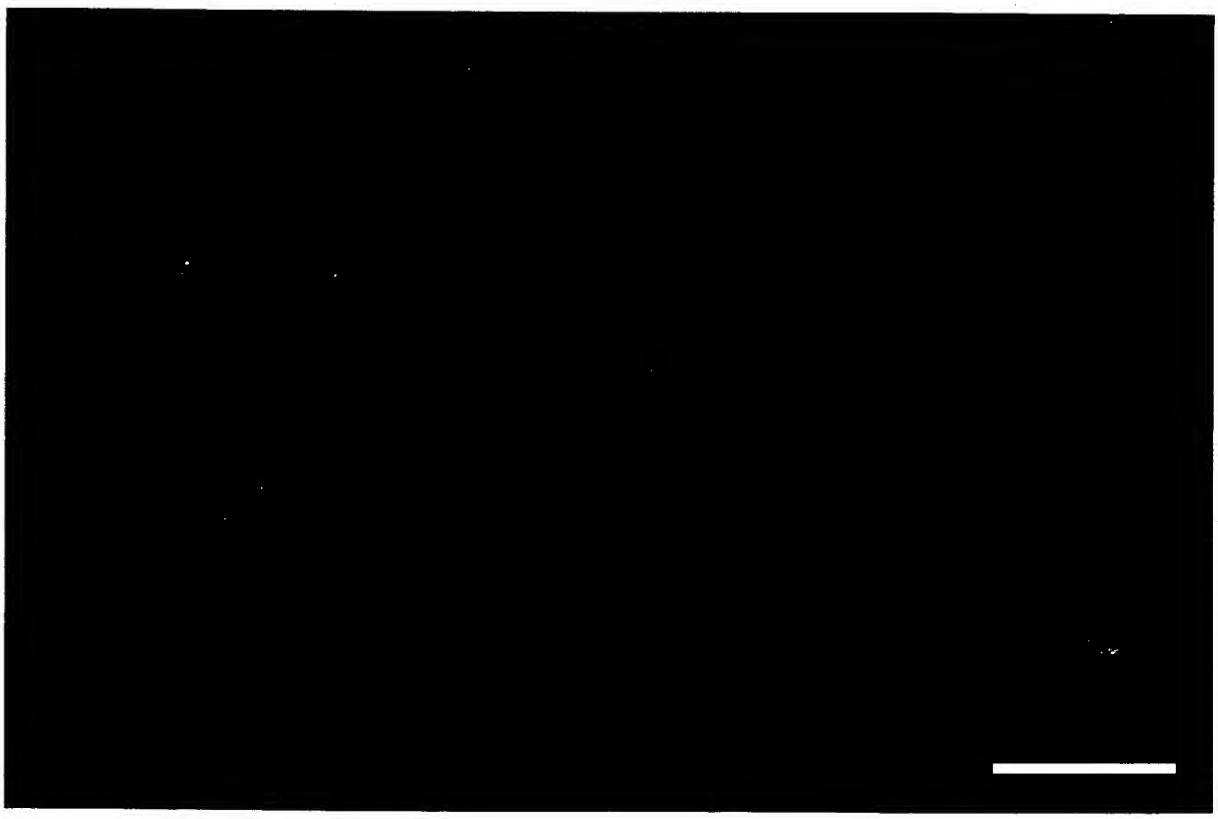
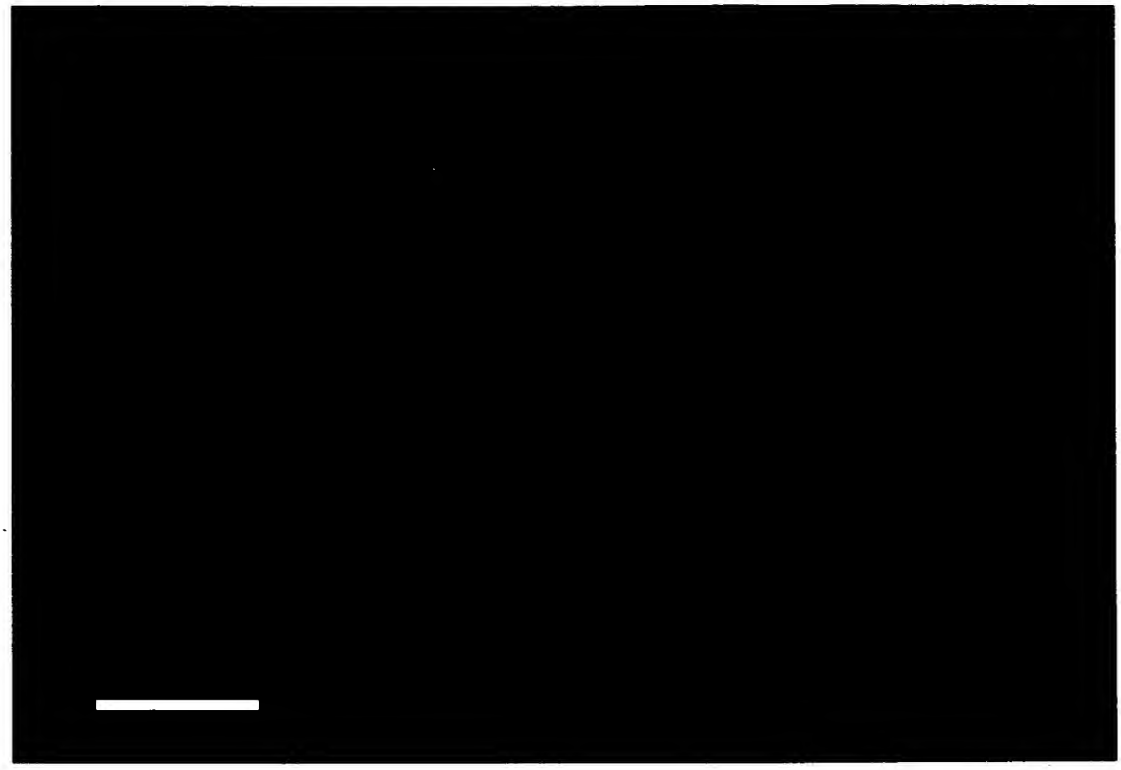
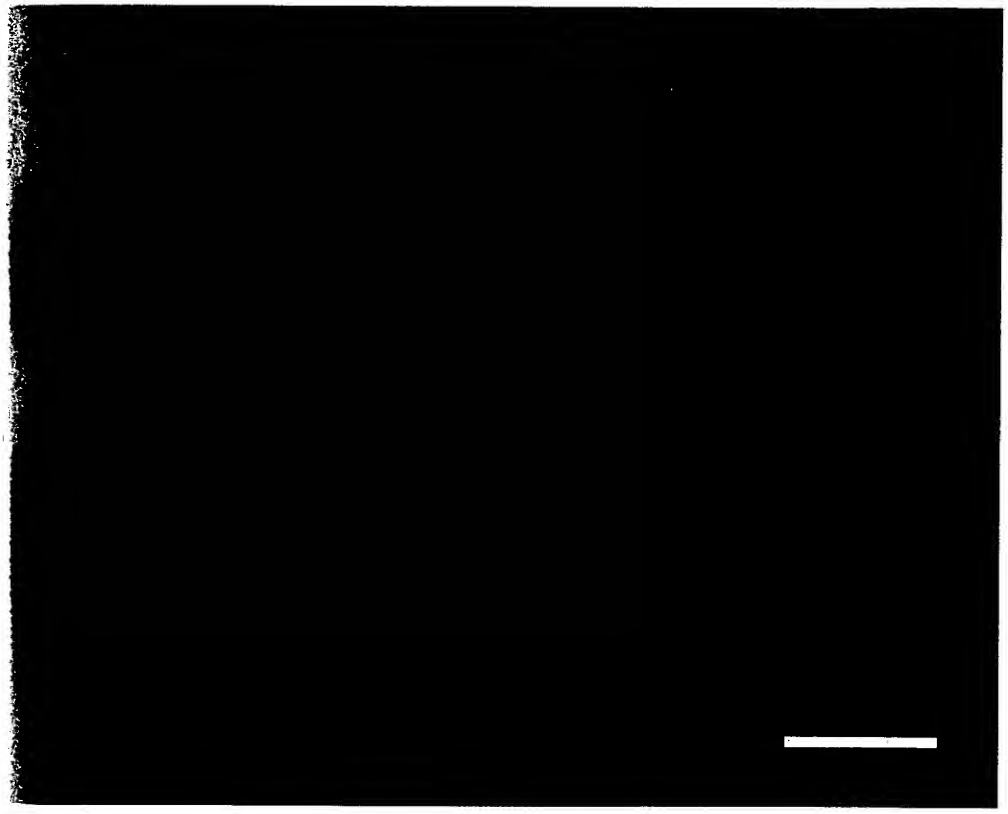


Figure 13





15: